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13. SUPPLEMENTARY NOTES

14. ABSTRACT The basal-like breast cancer (BLBC) subtype accounts for a high percentage of overall breast cancer mortality. The current therapeutic options for BLBC need improvement; hence, elucidating signaling pathways that drive BLBC growth may identify novel targets for the development of effective therapies. Rho GTPases have previously been implicated in promoting tumor cell proliferation and metastasis. These proteins are inactivated by GTPase-activating proteins (GAPs), which have generally been presumed to act as tumor suppressors. Surprisingly, RNA-Seq analysis of the Rho GTPase signaling transcriptome revealed high expression of several RhoGAP genes in BLBCs. The aim of our research is to characterize the role of two of these RhoGAPs, ArhGAP11A and RacGAP1, in BLBC development. Both proteins were highly expressed in human BLBC cell lines, and knockdown of either gene resulted in significant defects in the proliferation of these cells. Knockdown of ArhGAP11A caused CDKN1B/p27-mediated cell cycle arrest, whereas RacGAP1-depletion inhibited growth through the combined effects of cytokinesis failure, CDKN1A/p21-mediated RB1 inhibition, and the onset of senescence. Random migration was suppressed or enhanced by the knockdown of ArhGAP11A or RacGAP1, respectively. Cell spreading and levels of GTP-bound RhoA were increased upon depletion of either GAP. We have established that ArhGAP11A and RacGAP1 are both critical drivers of BLBC growth, and propose that RhoGAPs can act as oncogenes in cancer.

15. SUBJECT TERMS
ArhGAP11A, RacGAP1, basal-like breast cancer, RhoGAPs, Rho GTPases, RhoA, proliferation, migration

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1. INTRODUCTION:

The basal-like subtype of human breast cancer accounts for a disproportionately high percentage of overall breast cancer recurrence and death, and the current therapeutic options for this cancer need improvement. Hence, elucidating the signaling pathways that are responsible for driving the growth of basal-like tumors may identify novel targets for the development of effective therapies. Rho family small GTPases have previously been implicated in promoting tumor cell proliferation, invasion, and metastatic growth in a variety of cancers. These proteins are activated by guanine nucleotide exchange factors (GEFs) and, in the context of cancer, overexpressed RhoGEFs can function as oncogenes which cause hyper-elevated Rho GTPase activity. In contrast, GTPase-activating proteins (GAPs), which return Rho GTPases to an inactive, GDP-bound state, have generally been presumed to act as tumor suppressors. Surprisingly, microarray analysis of the expression of Rho GTPases, GEFs, and GAPs across a panel of human breast tumors revealed that a number of RhoGAP genes were significantly upregulated in basal-like breast cancers (BLBCs). These preliminary results suggested that RhoGAPs may play an unexpected role in promoting tumor growth. The aim of our research was therefore to validate and characterize the role of two of these RhoGAPs, ArhGAP11A (also known as MP-GAP) and RacGAP1 (also known as MgcRacGAP and CYK4), in BLBC development.

2. KEYWORDS: ArhGAP11A, RacGAP1, basal-like breast cancer, RhoGAPs, Rho GTPases, RhoA, proliferation, migration

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project, as described in the approved Statement of Work, were as follows:

Major Task 1. Validate the role of RhoGAPs in BLBC oncogenesis

a) *In vitro* tumor growth and invasion assays.
b) Western blots for ArhGAP11A and RacGAP1 protein expression in human tumor samples, human cell lines, and mouse models.
c) *In vivo* tumorigenesis and metastasis assays.

Milestones: Identify whether ArhGAP11A and RacGAP1 can promote tumor growth and/or invasion both *in vitro* and *in vivo* (by month 12). **Approximately 90% completed by month 24.**

Major Task 2. Determine the functions of ArhGAP11A and RacGAP1

a) Proliferation, cytokinesis, and apoptosis assays.
b) Transformation assays.
c) Migration analyses.

Milestones: Identify the specific functions of ArhGAP11A and RacGAP1 in BLBC tumorigenesis or metastasis (by month 24). **Completed by month 24.**

Major Task 3. Determine if ArhGAP11A and RacGAP1 promote tumorigenesis in a GAP activity-dependent manner

a) Express and purify isolated GAP domains and catalytically-inactive mutants of ArhGAP11A and RacGAP1.

b) GAP activity assays.
c) Rho GTPase pulldown assays.
d) Rescue experiments with wild type or catalytically-inactive GAPs.

Milestones: Identify the GTPase specificity of ArhGAP11A and determine if disrupted GTPase activity is responsible for the tumorigenic phenotypes of ArhGAP11A- or RacGAP1-depleted cells (by month 36). **Approximately 90% completed by month 24.**

What was accomplished under these goals?

- **Major Activities and Specific Objectives**

The major research activities undertaken in this project were to validate the role of ArhGAP11A and RacGAP1 in BLBC oncogenesis, to determine the function of these RhoGAPs in BLBC cell lines, and to identify the signaling pathways involved. SUM149 and HCC1937 were identified to be appropriate human BLBC cell lines to use for this study, as both exhibited high protein expression levels of the two RhoGAPs in question. Protocols to efficiently knockdown ArhGAP11A or RacGAP1 from these cell lines, using lentivirally-delivered shRNA constructs, were then established. Successful depletion of these proteins allowed us to pursue the major objective of identifying whether loss of expression of either ArhGAP11A or RacGAP1 in BLBC cell lines led to changes in cellular function that are consistent with a role for these GAPs in promoting BLBC tumorigenesis. More specifically, we assessed the ability of SUM149 and HCC1937 cell lines to proliferate in vitro in the absence of these GAPs (using 2D clonogenic and/or MTT proliferation assays) and, having identified a growth defect, went on to characterize the mechanisms through which growth was inhibited in either case, by performing apoptosis, cytokinesis, cell cycle, and senescence assays. In addition, fluorescent microscopy and time-lapse imaging were used to identify defects in the ability of ArhGAP11A- and RacGAP1-depleted cells to spread and migrate. To assess the effect of GAP knockdown on Rho GTPase signaling, we performed Rho GTPase pulldown assays. Finally, we overexpressed ArhGAP11A and RacGAP1 in MCF10A breast myoepithelial cells to identify whether these GAPs were capable of transforming untransformed immortalized cells.

- **Key Outcomes**

Reference and figure numbers refer to those found in Appendix 1, which is a manuscript of this research accepted for publication by Cancer Research (acceptance letter is found in Appendix 2).

To identify components of Rho GTPase signaling networks that are upregulated in BLBC, we analyzed The Cancer Genome Atlas (TCGA) Project (28) RNA-Seq data from 1,201 human breast tumors for the expression of the 20 Rho GTPase, 79 RhoGEF, 64 RhoGAP, and three RhoGDI genes across different breast cancer subtypes. Notably, several RhoGAP genes were found to be highly expressed in basal-like tumors relative to tumors of the normal-like (Fig. 1A) or luminal A (Fig. 1B) subtypes, which have a better prognosis. To explore the possibility that RhoGAP genes may play oncogenic roles in BLBC, we chose to focus on ARHGAP11A and RACGAP1, two GAP genes that were among the most highly upregulated Rho GTPase regulators in the basal-like subtype (Fig. 1A and B). At the protein level, ArhGAP11A and RacGAP1 expression was higher in BLBC cell lines, relative to cell lines of other subtypes (Fig. 1D, Supplementary Fig. 2A). Since SUM149 and HCC1937 cells expressed relatively high protein levels of both ArhGAP11A and RacGAP1, we elected to use these two BLBC cell lines in our functional studies to validate a role for these GAPs in BLBC tumorigenesis.

To assess the contribution of ArhGAP11A and RacGAP1 to BLBC growth, we first stably depleted these genes from SUM149 and HCC1937 cells using lentivirally-delivered shRNA. Western blot analyses indicated that protein expression of ArhGAP11A was reduced in both cell lines by ~90% and ~60% by the sh3 and sh5 vectors, respectively, relative to a non-silencing (NS) control (Fig. 2A and B, Supplementary Fig. S1). For RacGAP1, sh1 and sh2 constructs both caused ~90% knockdown (Fig. 2A and B, Supplementary Fig. S1). We then subjected cells to clonogenic growth assays. SUM149 or HCC1937 cells lacking either ArhGAP11A or RacGAP1 formed ~70-95% fewer colonies relative to the NS control (Fig. 2C and D). Similarly, in MTT viability assays, near-complete suppression of proliferation was observed in the absence of either GAP (Fig. 2E and F), suggesting that both GAPs are required for the efficient growth of these BLBC cell lines. These results therefore support the hypothesis that these RhoGAPs are playing an oncogenic role in BLBC cells.

The next step in identifying the function(s) of ArhGAP11A and RacGAP1 was to delineate the mechanism(s) through which each GAP supported BLBC proliferation. This was accomplished by performing apoptosis, cytokinesis, cell cycle, and senescence assays. Depletion of ArhGAP11A or RacGAP1 from SUM149 or HCC1937 cells did not result in detectable levels of cleaved PARP1, a marker for cell death (Fig. 2G and H). Hence, apoptosis is not responsible for causing the proliferation defect that is characteristic of ArhGAP11A- and RacGAP1-depleted cells.

RacGAP1 is known to regulate cytokinesis (29,30) and ArhGAP11A has also recently been implicated in the control of this process (31). To investigate the possibility that defects in cytokinesis may be responsible for the inability of either ArhGAP11A- or RacGAP1-depleted cells to proliferate, we examined the ability of GAP-deficient SUM149 or HCC1937 cells to efficiently divide. Defects in cytokinesis result in the formation of bi- or multinucleated cells, which were identified and quantitated using fluorescent microscopy. Consistent with the established role for RacGAP1 in regulating cytokinesis, ~30-40% of SUM149 cells and ~45% of HCC1937 cells became bi- or multinucleated upon RacGAP1 knockdown (Fig. 3A, B, and C). Hence, cytokinesis failure is likely to contribute to the inability of RacGAP1-depleted cells to proliferate. Although this slight defect may partially contribute to growth impairment, it is insufficient to account for the substantial growth defects observed upon knockdown of ArhGAP11A (Fig. 2C, D, E, and F).

Having ruled out apoptosis and cytokinesis failure as factors that make a major contribution to the growth defect of ArhGAP11A-deficient cells, we next performed flow cytometry analysis of propidium iodide-stained cells to identify whether GAP-deficient SUM149 arrest at a specific phase of the cell cycle. This analysis revealed that, relative to NS cells, ArhGAP11A-deficient cells accumulated in the G1 phase of the cell cycle, as shown by the decreased proportion of cells that entered the S or G2/M phases (Fig. 4A). The finding that ArhGAP11A is required for efficient cell cycle progression is indicative of a pro-tumorigenic role for this GAP.

We next performed western blot analyses for proteins that are involved in the G1 to S phase cell cycle transition, with the aim of elucidating the molecular mechanisms responsible for causing G1 arrest in ArhGAP11A-depleted cells. Notably, phosphorylation and inactivation of the RB1 tumor suppressor protein, which allows G1 to S progression by releasing the inhibition of E2F transcription factors, was dramatically reduced upon knockdown of ArhGAP11A (Fig. 4B). Phosphorylation of RB1 is controlled by cyclin-dependent kinases (CDKs) in complex with cyclins D1 and E, but can be prevented by CDK inhibitors such as CDKN2A/p16, p21, or p27. High expression of p27 was detected in lysates of SUM149 cells depleted of ArhGAP11A (Fig. 4B). As neither p21 (Fig. 4B) nor p16 (which is not expressed in SUM149 cells) were detected under the same conditions, these results indicate that p27 is the CDK inhibitor responsible for the hypophosphorylation of RB1 and the associated G1 arrest that

occurs upon ArhGAP11A knockdown. We suggest that p27-mediated arrest is likely to be the major mechanism through which ArhGAP11A-depleted cells fail to proliferate.

In contrast to ArhGAP11A, knockdown of RacGAP1 from SUM149 cells did not alter the cell cycle profile relative to control cells (Fig. 4A). Despite this, RacGAP1-depleted cells also had very low levels of RB1 phosphorylation (Fig. 4B). In these cells, the CDK inhibitor p21 was upregulated (Fig. 4B). As p21-mediated inhibition of RB1 phosphorylation is a pathway known to induce senescence (33), we next tested to see whether senescence may contribute to the growth defect of RacGAP1-depleted cells. Indeed, RacGAP1-deficient SUM149 and HCC1937 cells were found to have enhanced levels of senescence-associated β-galactosidase expression (Fig. 4C, D, and E). In contrast, ArhGAP11A-knockdown did not induce senescence (Fig. 4C, D, and E), providing further support for the distinct roles that ArhGAP11A and RacGAP1 play in BLBC cells: depletion of ArhGAP11A leads to decreased growth via p27-mediated cell cycle arrest, whereas RacGAP1-deficient cells fail to proliferate as a result of the combined effects of cytokinesis failure, p21-induction, and the onset of senescence.

In addition to studying the effects of ArhGAP11A and RacGAP1 on BLBC proliferation, we have examined the role of these GAPs in regulating cell spreading and migration, two processes that are important for invasive and metastatic cancer growth and that are known to be reliant on Rho GTPase-dependent cytoskeletal dynamics (34). The depletion of either ArhGAP11A or RacGAP1 caused mononucleated SUM149 cells to spread on fibronectin with an approximately 30-50% larger area than that of control cells, as assessed using fluorescent microscopy (Fig. 5A). Enhanced spreading was also observed on uncoated glass coverslips (Supplementary Fig. S3). As bi/multinucleated cells typically exhibit greatly increased spread areas, these cells were excluded from the spreading analysis. These results suggest that both ArhGAP11A and RacGAP1 are involved of the regulation of cell spreading, which would indicate that one of their functions may be to regulate the cytoskeleton via Rho GTPase signaling. However, the possibility that the spread morphologies of these cells may be secondarily linked to the onset of senescence (particularly in the case of RacGAP1-depleted cells) cannot be excluded.

The random migration of GAP-depleted SUM149 cells on fibronectin was assessed using time-lapse microscopy, in collaboration with Dr. James Bear's lab at UNC. Tracking the movement of individual cells over a 24 h period determined that SUM149 cells treated with ArhGAP11A sh3 had a significantly (~45%) reduced mean velocity compared to that of NS cells (Fig. 5B). This migratory defect was not observed upon treatment with the ArhGAP11A sh5 construct, most likely reflecting the enhanced efficiency of ArhGAP11A knockdown with sh3 compared to sh5 (Supplementary Fig. S1I). The mean migration velocity of RacGAP1-depleted cells was 32-58% faster than that of NS cells (Fig. 5B), indicating that ArhGAP11A promotes, whereas RacGAP1 inhibits, BLBC cell migration in vitro.

As Rho GTPases are known to directly regulate proliferation, cytokinesis, cell cycle progression, spreading, and migration – processes that we have shown to be affected by ArhGAP11A and/or RacGAP1 in BLBC cells, we next set out to determine which specific Rho GTPases are controlled by these two GAPs. In SUM149 cells, pulldown experiments for active GTPases revealed that RhoA was 43-82% more active upon depletion of ArhGAP11A than in control cells (Fig. 6A and B). Rac1 and Cdc42 activity were unaffected by knockdown of this GAP. These results are consistent with ArhGAP11A having catalytic GAP activity toward RhoA, as has been demonstrated using in vitro assays (31,35,36). In contrast, previous work has demonstrated that RacGAP1 acts as a GAP for Rac1 and Cdc42, but not RhoA in vitro (37). Surprisingly, Rho GTPase pulldown experiments showed that RhoA activity, but not that of Rac1 or Cdc42, was elevated in SUM149 cells upon depletion of RacGAP1 (Fig. 6A and B). These results suggest that both ArhGAP11A and RacGAP1 usually suppress RhoA activity in BLBC cells. To assess whether increased RhoA activity is responsible for the

proliferation defect of ArhGAP11A- or RacGAP1-depleted cells, we performed clonogenic growth assays in the presence Y-27632, an inhibitor of the Rho effector ROCK. ROCK inhibition led to a partial rescue of the growth defect of ArhGAP11A or RacGAP1-depleted cells (Fig. 6C), indicating that restricted RhoA signaling is required for efficient proliferation. Furthermore, transfection of SUM149 cells with a constitutively active RhoA mutant (Q63L) resulted in decreased proliferation (Fig. 6D and E). These results indicate that persistently elevated RhoA activity is prohibitive to BLBC growth.

To assess the effect of ArhGAP11A and RacGAP1 on transformation, we stably overexpressed these proteins in untransformed immortalized human MCF10A breast myoepithelial cells (Fig. 7A). In MTT viability assays, ArhGAP11A expression resulted in enhanced MCF10A proliferation, as did expression of a known oncogene, mutationally-activated KRAS4B(G12V) (Fig. 7B). These data support the idea that ArhGAP11A can act as a cancer driver. Interestingly, RacGAP1 overexpression did not cause an increase in proliferation relative to the control (Fig. 7B). It is possible that other signaling components must also be altered for RacGAP1 to enhance proliferation. We next performed MCF10A acinar formation assays to evaluate the effect of RhoGAP overexpression on mammary epithelial morphogenesis. Expression of oncogenes in MCF10A cells can lead to the formation of disrupted acinar morphology; for example, KRAS4B(G12V) expression resulted in the formation of large multi-acinar structures (Fig. 7C). Acini ectopically overexpressing either ArhGAP11A or RacGAP1 exhibited a disrupted, less spherical architecture relative to control acini as early as 8 days after plating (Fig. 7C and D). This is consistent with an oncogenic effect for both ArhGAP11A and RacGAP1.

In summary, the research performed under this award has produced several key outcomes with respect to validating and characterizing the functions of ArhGAP11A and RacGAP1 in BLBC. Both of these RhoGAPs are highly expressed in BLBC and are essential for the proliferation of basal-like cell lines. Knockdown of ArhGAP11A causes p27-mediated arrest in the G1 phase of the cell cycle, whereas depletion of RacGAP1 inhibits growth through the combined effects of cytokinesis failure, p21-mediated RB1 inhibition, and the onset of senescence. Random migration is suppressed or enhanced by the knockdown of ArhGAP11A or RacGAP1, respectively, whereas cell spreading and levels of GTP-bound RhoA are increased upon depletion of either GAP. We have established that ArhGAP11A and RacGAP1 are both critical drivers of BLBC growth, and propose that RhoGAPs can act as oncogenes in cancer.

- **Other Achievements**

The above research was accepted for publication in Cancer Research in April 2016 (see attached manuscript in Appendix 1 and acceptance letter in Appendix 2). Other accomplishments under this award include the publication of a review paper in the journal Small GTPases in March 2014. This review focused on the role of GAPs and GEFs in regulating RhoA- and Rac1-mediated cellular adhesion and migration – concepts that are intimately linked to the above research. The review reference is: Lawson CD, Burridge K. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. Small GTPases 2014; 5:e27958 (PMID 24607953). Support from the DoD was acknowledged.

I also attended three research conferences over the last two years, which afforded me invaluable opportunities to present my data and allowed me to meet and discuss my research with relevant people in the field. In June 2015, I attended the 'Regulation and Function of Small GTPases' FASEB Summer Research Conference and my abstract was selected by the organizers to give a 15 min presentation. DoD support was acknowledged. In December 2014, I attended the annual ASCB conference in Philadelphia, PA and presented a poster on my research. The poster abstract reference is: Lawson CD, Rossman KL, Fan C, Perou CM, Burridge K, Der CJ. The role of RhoGAPs in basal-like breast cancer. Mol. Biol. Cell

2014; 25:185-186 (Abstract P1855). Again, DoD support was acknowledged. I also attended 'The Triangle Cytoskeleton Meeting,' an ASCB local meeting held at the Research Triangle Park, NC in September 2014.

What opportunities for training and professional development has the project provided?

Under the co-mentorship of Drs. Der and Burridge, and with the help of their respective lab members and other researchers at UNC, this project exposed me to new techniques, skills, and equipment and therefore provided excellent training opportunities. Furthermore, I actively engaged in other activities (e.g. seminars, lab meetings, individual study) that enhanced my professional development.

The diverse research interests of the Der and Burridge labs allowed me to interact daily with experts in particular topics and techniques and I exploited the respective knowledge of each lab to receive one-on-one training that greatly contributed to this project. For example, I received training in performing in vitro growth assays (2D clonogenic, MTT, senescence, acinar) from experienced members of the Der lab, whereas members of the Burridge lab tutored me in the performance of Rho GTPase pulldown assays as well as in fluorescent microscopy techniques. Training in the acquisition of fluorescent images was also provided by a regional representative from Zeiss. In addition to members of the Der and Burridge labs, I received training from other UNC-based researchers and facility staff. Notably, I was taught how to perform time-lapse imaging by a graduate student in Dr. James Bear's lab using their Olympus Vivaview system. I also received training from staff at UNC's Flow Cytometry Core Facility that has allowed me to perform cell cycle analyses.

The research environment at UNC provided not only excellent opportunities for training, but also a high-caliber platform for professional development in the form of regular lab meetings, journal clubs, seminars, and visiting lecturers. At the lab level, the Der and Burridge labs both hold weekly lab meetings/journal clubs and these provided me with the chance to learn about my peers' research and to gain knowledge of their particular interests. Furthermore, I presented my own research to both labs approximately every three months, which provided constructive feedback and helped me to develop my presentation skills. I also presented my research at a departmental level. Departmental seminars are held weekly and allowed me to learn about other people's research at UNC. Seminars presented by visiting lecturers also occur frequently and afforded useful insight into work being carried out at other institutions. Interacting with guest speakers, coupled to my attendance at three conferences (as described above), provided me with the opportunity to form professional contacts on a local, national, and international level. Finally, through individual study I gained knowledge of new areas that were important to my research and which complemented my pre-existing expertise.

How were the results disseminated to communities of interest?

These results will be disseminated to the research community through their publication in Cancer Research, where the attached manuscript was recently accepted. They were also conveyed to other researchers in the field at the 2015 'Regulation and Function of Small GTPases' FASEB Summer Research Conference, where I gave an oral presentation. Furthermore, I presented my data as a poster at the 2014 annual ASCB conference.

In addition to communication with other researchers within the field, I have also been involved with outreach activities to reach a more public audience. Notably, I have participated in 'open lab' events in which members of the public – largely cancer patients, survivors, affected families, and advocates – have been invited to tour the Der lab facilities and hear about the research that we do. Communication of

our research to a lay audience in this manner is a critical way in which to increase public understanding, but also serves as an important reminder of the relevance of our research to the wider community.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Once published, the results of this project are likely to impact upon the field of cancer biology in two key ways. Firstly, by reclassifying RhoGAPs as a class of molecule that can support oncogenesis, and secondly, by establishing ArhGAP11A and RacGAP1 as potential targets for pharmacological intervention in the treatment of BLBC.

Rho family small GTPases have been strongly linked to tumor growth and metastasis, typically through their aberrant activation by GEFs, which is generally thought to result in hyper-elevated Rho GTPase activity and therefore tumorigenesis. In contrast, RhoGAPs, which downregulate Rho GTPase activity and are relatively understudied in comparison to RhoGEFs, are generally presumed to act as tumor suppressors. The results of our study, which indicate that ArhGAP11A and RacGAP1 are overexpressed in BLBC and are essential for tumor growth, are contrary to the perceived notion that RhoGAPs can only act as tumor suppressors and indicate that certain GAPs can in fact promote tumorigenesis. These surprising findings should provoke a reassessment of the role of RhoGAPs in human cancer and may lead to additional studies with the potential to identify other RhoGAPs as drivers of cancer.

By identifying, validating, and characterizing ArhGAP11A and RacGAP1 as oncogenes in the development of BLBC tumor growth, our research has defined these proteins as novel molecular targets for the development of therapeutic strategies, with the potential to improve treatment of patients with this particularly aggressive subtype of breast cancer. Hence, our research will not only contribute to knowledge of our particular field but may also act as the basis for the development of improved subtype- and molecularly- targeted cancer therapies.

What was the impact on other disciplines? Nothing to report.

What was the impact on technology transfer? Nothing to report.

What was the impact on society beyond science and technology? Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.
Lawson CD, Fan C, Mitin N, Baker NM, George SD, Graham DM, Perou CM, Burridge K, Der CJ, Rossman KL. Rho GTPase transcriptome analysis reveals oncogenic roles for Rho GTPase-activating proteins in basal-like breast cancers. Cancer Res, in press (accepted 22 April 2016).
(Accepted, federal support acknowledged)

Lawson CD, Burridge K. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. Small GTPases 2014; 5:e27958.
(Published, federal support acknowledged)

Lawson CD, Rossman KL, Fan C, Perou CM, Burridge K, Der CJ. The role of RhoGAPs in basal-like breast cancer. Mol. Biol. Cell 2014; 25:185-86 (Abstract P1855).
(Published abstract, federal support acknowledged)

Books or other non-periodical, one-time publications. Nothing to report.

Other publications, conference papers, and presentations.
Lawson CD, Rossman KL, Graham DM, Fan C, Perou CM, Burridge K, Der CJ. Paradoxical driver roles of the ArhGAP11A and RacGAP1 RhoGAPs in basal-like breast cancer. Presented at FASEB Regulation and Function of Small GTPases meeting, June 7-12 2015, West Palm Beach, FL.
(federal support acknowledged)

- **Website(s) or other Internet site(s).** Nothing to report.
- **Technologies or techniques.** Nothing to report.
- **Inventions, patent applications, and/or licenses.** Nothing to report.
- **Other Products.** Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Campbell Lawson
Project Role:	PI
Nearest person month worked:	12
Contribution to Project:	All experiments and analysis
Funding Support:	This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Campbell Lawson (PI)
Nothing to report

Keith Burridge (Key personnel)
Grants awarded in the last 12 months:

1R21 AI113201-A1 (Burridge, Cerami, and Gomez)	08/01/15-07/31/17	1.2 calendar
NIAID	$125,000	
Endothelial Cell Uptake of Infected Erythrocytes in Cerebral Malaria		
2-U54-CA156733-06 (Earp (PI))	9/1/15 - 8/31/20	0.60 calendar
National Cancer Institute	$52,363.00	
Burridge (Co-project leader) Full Project 1: LSR Alters Metabolic Signaling to Drive Aggressive Breast Cancer Behaviors		

Channing Der (Key personnel)
Grants awarded in the last 12 months:

W81XWH-15-1-0611 (Der)	7/1/2015 - 6/30/2017	0.45 Calendar
Department of Defense	$224,211	
Targeting KRAS for Pancreatic Cancer Treatment		
15-90-25-DER (Der)	7/1/2015 - 6/30/2018	0.90 Calendar
Pancreatic Cancer Action Network-AACR	$490,621	
Defining Novel Combination KRAS-targeted Therapeutic Strategies		
1U01-CA199235-01 (Der, Cox)	9/1/2015 - 6/30/2019	1.2 Calendar
NIH/NCI	$571,853	
Identification of Synthetic Lethal Interactors in Pancreatic Cancer		

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

Appendix 1: Manuscript accepted for publication by Cancer Research
Appendix 2: Acceptance letter from Cancer Research

Rho GTPase Transcriptome Analysis Reveals Oncogenic Roles for Rho GTPase-activating Proteins in Basal-like Breast Cancers

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Abstract

The basal-like breast cancer (BLBC) subtype accounts for a disproportionately high percentage of overall breast cancer mortality. The current therapeutic options for BLBC need improvement; hence, elucidating signaling pathways that drive BLBC growth may identify novel targets for the development of effective therapies. Rho GTPases have previously been implicated in promoting tumor cell proliferation and metastasis. These proteins are inactivated by Rho-selective GTPase-activating proteins (RhoGAPs), which have generally been presumed to act as tumor suppressors. Surprisingly, RNA-Seq analysis of the Rho GTPase signaling transcriptome revealed high expression of several RhoGAP genes in BLBC tumors, raising the possibility that these genes may be oncogenic. To evaluate this, we examined the roles of two of these RhoGAPs, ArhGAP11A (also known as MP-GAP) and RacGAP1 (also known as MgcRacGAP), in promoting BLBC. Both proteins were highly expressed in human BLBC cell lines, and knockdown of either gene resulted in significant defects in the proliferation of these cells. Knockdown of ArhGAP11A caused CDKN1B/p27-mediated arrest in the G1 phase of the cell cycle, whereas depletion of RacGAP1 inhibited growth through the combined effects of cytokinesis failure, CDKN1A/p21-mediated RB1 inhibition, and the onset of senescence. Random migration was suppressed or enhanced by the knockdown of ArhGAP11A or RacGAP1, respectively. Cell spreading and levels of GTP-bound RhoA were increased upon depletion of either GAP. We have established that, via the suppression of RhoA, ArhGAP11A and RacGAP1 are both critical drivers of BLBC growth, and propose that RhoGAPs can act as oncogenes in cancer.

Introduction

Human breast tumors can be classified into one of five major subtypes (luminal A, luminal B, HER2-enriched, basal-like, and normal breast-like) based upon global gene expression analyses (1-3). The basal-like subgroup is responsible for a disproportionately high percentage of overall breast cancer recurrence and death, and accounts for approximately 80% of the 'triple-negative' breast cancers (4,5), for which the current standard of care is limited to conventional cytotoxic chemotherapy (6).

Rho family small GTPases (e.g., RhoA, Rac1, and Cdc42) are intracellular signaling molecules belonging to the Ras superfamily (7,8). When GTP-bound, these proteins are active and capable of signaling to a diverse array of downstream effectors, through which they regulate cellular responses such as cell cycle progression, cytokinesis, survival, migration, and polarity. Rho GTPases have been strongly implicated in tumorigenesis (9), yet, until recently, these proteins were rarely identified as being mutated in cancer. Instead, dysregulated activity of Rho GTPases in cancer more commonly arises through their aberrant expression and/or activation.

Rho GTPase activity is regulated by Rho-selective guanine nucleotide exchange factors (RhoGEFs), GTPase-activating proteins (RhoGAPs), and guanine nucleotide dissociation inhibitors (RhoGDIs). In the context of cancer, RhoGEFs, which promote the formation of the active GTP-bound state of Rho GTPases by catalyzing the exchange of GDP for GTP (10), are thought to drive tumor growth when overexpressed or aberrantly activated. For example, we recently showed that the RhoGEF PREX1 is overexpressed in melanoma and that PREX1-deficient mice have impaired metastatic tumor growth (11). In contrast, RhoGAPs, which stimulate the intrinsic GTPase activity of Rho proteins and return them to an inactive, GDP-bound state (12), are generally presumed to inhibit tumorigenesis (13). For example, the

RhoGAP DLC1 is commonly lost in cancer through promoter methylation, genomic deletion, or enhanced protein degradation (14,15). Hence, the prevailing dogma in the field is that Rho GTPases and RhoGEFs are oncogenes in cancer, whereas RhoGAPs are tumor suppressors.

Recent evidence has begun to challenge this notion, however. Genomic sequencing has revealed frequent somatic mutations of RhoA in peripheral T cell lymphomas (PTCLs) (16-18) and in diffuse-type gastric carcinomas (19-21). Surprisingly, unlike Rac1, which has gain-of-function mutations in melanoma (22), hotspot mutations in RhoA were identified at sites consistent with loss-of-function. In PTCL, the predominant mutation was G17V, which abolishes GTP-binding and causes RhoA to act as a dominant-negative inhibitor of RhoGEF activity (16-18). Diffuse-type gastric cancers exhibited mutations in the effector binding domain of RhoA, most commonly Y42C (19-21), which prevents binding to the Rho effector PKN1 (23). In addition to these mutational analyses, another recent study has shown that colorectal cancer growth is accelerated in the presence of dominant-negative (T19N) RhoA (24). Taken together, and contrary to the existing paradigm, this emerging evidence suggests that, in certain cancers, wild type RhoA may act as a tumor suppressor.

In this study, we analyzed RNA-Seq data of human breast tumors to identify Rho GTPases or GTPase regulators that are aberrantly expressed in breast cancer. To our surprise, several RhoGAP genes exhibited high expression in the basal-like subtype. Focusing on two of these genes, *ARHGAP11A* and *RACGAP1*, we characterized their role(s) in promoting basal-like breast cancer (BLBC). In basal-like cell lines, both GAPs were required for proliferation. Suppression of ArhGAP11A (also known as MP-GAP) expression caused cells to undergo cell cycle arrest, whereas RacGAP1 (also known as MgcRacGAP)-depleted cells failed to grow as a result of the combined effect of cytokinesis failure and the onset of senescence. We propose that

ArhGAP11A and RacGAP1 act as oncoproteins in BLBC and that GAP-mediated inhibition of RhoA activity is a pro-proliferative mechanism, consistent with the emerging view of RhoA as a tumor suppressor.

Materials and Methods

Cell Culture

Cells were cultured at 37°C in a humidified/5% CO_2 atmosphere. SUM149 and SUM159 cells (Asterand) were maintained in Ham's F-12 medium (Gibco), 10 mM HEPES, 5 μg/ml insulin, 1 μg/ml hydrocortisone, and 5% FBS. HMLE cells (provided by Robert Weinberg, MIT) were cultured in mammary epithelial cell growth medium (Lonza). BT474, HCC1937, HEK293T, MCF10A, MCF7, MDA-MB-231, MDA-MB-468, and T47D cells were purchased from American Type Culture Collection (ATCC) and maintained according to ATCC instructions. All cell lines were obtained between 2003 and 2015 and passaged for fewer than 6 months after receipt/resuscitation from cell banks. For apoptosis experiments, SUM149 or HCC1937 cells were treated with 1 μM staurosporine for 4 or 6 h, respectively.

Vectors and lentivirus preparation

Target short hairpin RNA (shRNA) sequences (in pLKO.1 vectors) were as follows: non-silencing (NS) control 5'-CAACAAGATGAAGAGCACCAA-3', ArhGAP11A sh3 5'-CTGGTGTCAATAGATATGAAA-3', ArhGAP11A sh5 5'-CCTTCTATTACACCTCAAGAA-3', RacGAP1 sh1 5'-CAGGTGGATGTAGAGATCAAA-3', and RacGAP1 sh2 5'-CTAGGACGACAAGGCAACTTT-3'. A cDNA clone containing the ORF of *ARHGAP11A* (Genbank accession NM_014783) was generated by subcloning bp 2204-3794 of exon 12 onto

the 3' end of IMAGE clone 5502381 (Genbank accession BC063444, Center for Cancer Systems Biology), using an engineered XhoI site and partial overlapping primers to excise intervening sequences. Full-length *RACGAP1* cDNA was from the Center for Cancer Systems Biology (IMAGE clone 5583315, Genbank accession BC032754). ArhGAP11A, RacGAP1, and KRAS4B(G12V) cDNAs were subcloned into the pCDH-HA lentiviral vector (System Biosciences). Lentivirus particles were produced by transfecting HEK293T cells with target vectors and the ViraPower lentiviral packaging system (ThermoFisher Scientific). For lentiviral transduction, cells were incubated with virus for 14 h, passaged 48 h after initial transduction, and then used in experiments after puromycin selection. We have previously described the pCMV-Myc-RhoA Q63L, pCMV-Myc-Rac1 Q61L, and pCMV-Myc-Cdc42 Q61L vectors (25).

Immunoblot analyses

Antibodies recognizing the following proteins were used: ArhGAP11A (Abcam, ab113261, 1:1,000), RacGAP1 (Abnova, H00029127-M01, 1:1,000), Rac1 (BD Biosciences, 610650, 1:500), HA epitope tag (BioLegend, 901513, 1:500), cyclin D1 (Cell Signaling, 2922, 1:1,000), MAPK1/3 (ERK; Cell Signaling, 9102, 1:1,000), p21 (Cell Signaling, 2947, 1:1,000), p27 (Cell Signaling, 2552, 1:1,000), PARP1 (Cell Signaling, 9542, 1:3,000), phospho-ERK (Cell Signaling, 4370, 1:1,000), phospho-RB1 (Cell Signaling, 9308, 1:1,000), phospho-RPS6KA1 (RSK; Cell Signaling, 9344, 1:1,000), RB1 (Cell Signaling, 9309, 1:1,000), RhoA (Cell Signaling, 2117, 1:1,000), actin (EMD Millipore, MAB1501, 1:10,000), Cdc42 (Santa Cruz, sc-87, 1:200), cyclin E1 (Santa Cruz, sc-247, 1:500), and TP53 (Santa Cruz, sc-6243, 1:5,000). Densitometric quantification of blots was performed using ImageJ software (NIH).

Growth assays

For anchorage-dependent clonogenic growth assays, 10^4 cells/well were grown in 6-well plates in the presence or absence of 10 μM of the ROCK1/2 inhibitor Y-27632 (EMD Millipore, 688000) for 7 (SUM149) or 10 (HCC1937) days, then stained with 0.2% crystal violet in 4% formaldehyde for 20 min. For MTT viability assays, 1,000 cells/well (500 cells/well for SUM149) were grown in 96 well plates for up to 14 days then stained with 0.3 mg/ml MTT for 3 h. After solubilizing in dimethyl sulfoxide, A_{550} was recorded using a BioTek Synergy 2 plate reader. MCF10A acinar formation assays were performed as previously described (26). For fluorescent microscopy, acini were fixed after 12 days then stained with Alexa Fluor 568 phalloidin (ThermoFisher Scientific, A12380, 1:250) and Hoechst 33342 (ThermoFisher Scientific, H3570, 1:10,000). Images were taken using a Zeiss Axiovert 200M microscope (10× objective), Hamamatsu ORCA-ER camera, and Axiovision software. Acinar perimeter and area were determined using ImageJ software (NIH).

Cell cycle analysis

Cells were fixed in 70% ethanol for at least 30 min, stained with 50 μg/ml propidium iodide in PBS plus 100 μg/ml RNase for 15 min at 37°C, then analyzed for DNA content using a CyAn ADP flow cytometer and Summit software (Beckman Coulter).

Senescence

Senescence-associated β-galactosidase was detected 7 days post-plating using a staining kit (Cell Signaling, 9860), according to the manufacturer's instructions. Images were taken using a Nikon Eclipse TS100 microscope (20× objective) and Apple iPhone 6 camera.

Spreading assays and fluorescence microscopy

Cells were suspended in Ham's F-12 media, 10 mM HEPES, 5 μg/ml insulin, 1 μg/ml hydrocortisone, and 0.5% fatty acid-free bovine serum albumin, rotated end-over-end at 37°C for 1 h, then plated on 10 μg/ml fibronectin-coated coverslips for 2 h. For fluorescent microscopy, cells were fixed and then stained with Alexa Fluor 488 phalloidin (ThermoFisher Scientific, A12379, 1:500) and Hoechst 33342 (ThermoFisher Scientific, H3570, 1:10,000). Images were taken using a Zeiss Axiovert 200M microscope (63× objective), Hamamatsu ORCA-ER camera, and Axiovision software. Cell area was determined using ImageJ software (NIH); multinucleated cells were excluded from the spreading analysis.

Random migration assay

Cells (5×10^4/well) were plated on 10 μg/ml fibronectin in 35 mm MatTek dishes and imaged at 37°C, 5% CO_2 every 10 min for 24 h using an Olympus VivaView system (10× objective). Random migration was analyzed using the ImageJ software (NIH) Manual Tracking plugin, and the Ibidi Chemotaxis and Migration Tool.

Rho GTPase pulldowns

GTP-bound RhoA, Rac1, and Cdc42 levels were measured as we have previously described (27).

Results

ArhGAP11A and RacGAP1 are highly expressed in BLBC

To identify components of Rho GTPase signaling networks that are upregulated in BLBC, we analyzed RNA-Seq data, coming from 1,201 human breast tumors as part of The Cancer Genome Atlas (TCGA) Project (28), for the expression of the 20 Rho GTPase, 79 RhoGEF, 64 RhoGAP, and three RhoGDI genes across different breast cancer subtypes. Strikingly, a number of genes encoding RhoGAPs were found to be highly expressed in tumors of the basal-like subtype relative to normal-like (Fig. 1A) or luminal A (Fig. 1B) tumors, which have a better prognosis. This was surprising, given that RhoGAPs have been generally presumed to act as tumor suppressors. To explore the possibility that RhoGAP genes may in fact play oncogenic roles in BLBC, we chose to focus on two GAP genes that were among the most highly upregulated Rho GTPase regulators in the basal-like subtype, *ARHGAP11A* and *RACGAP1* (Fig. 1A and B). Similarly to the mRNA levels of these genes in human tumors (Fig. 1C), protein expression of ArhGAP11A and RacGAP1 in human breast cancer cell lines was generally higher in those of the basal-like subtype than in other subtypes (Fig. 1D, Supplementary Fig. 2A). The basal-like cell lines SUM149 and HCC1937 both exhibited high expression of ArhGAP11A and RacGAP1 (Fig. 1D), and we therefore used these cell lines to determine the biological function(s) of these proteins in BLBC.

ArhGAP11A and RacGAP1 are both required for BLBC cell line proliferation

To assess the contribution of ArhGAP11A and RacGAP1 to BLBC cell growth, we stably suppressed their expression in SUM149 and HCC1937 cells using two separate lentivirus-delivered shRNA constructs per gene. In both cell lines, steady-state ArhGAP11A protein expression was consistently reduced by ~90% and ~60% by the sh3 and sh5 vectors, respectively, relative to NS or uninfected (labelled as SUM149 or HCC1937) control cells (Fig.

2A and B, Supplementary Fig. S1). For RacGAP1, sh1 and sh2 constructs both caused ~90% knockdown (Fig. 2A and B, Supplementary Fig. S1).

We then performed a clonogenic growth assay to determine the effect of reduced ArhGAP11A or RacGAP1 expression on anchorage-dependent proliferation. SUM149 or HCC1937 cells depleted of ArhGAP11A or RacGAP1 formed ~70-95% fewer colonies of proliferating cells relative to the NS control (Fig. 2C and D). Near-complete suppression of proliferation was also observed for each RhoGAP using an MTT viability assay (Fig. 2E and F). The failure of BLBC cell lines to proliferate upon depletion of ArhGAP11A or RacGAP1 suggests that these GAPs act to promote cancer cell growth. Therefore, our results support the hypothesis that ArhGAP11A and RacGAP1 have oncogenic, rather than tumor suppressive, roles in BLBC cells. Depletion of these proteins from HER2-enriched (BT474) or luminal B (MCF7 and T47D) human breast cancer cell lines also caused defective proliferation (Supplementary Fig. S2), indicating that the importance of ArhGAP11A and RacGAP1 to breast cancer growth is not restricted to the BLBC subtype alone.

RacGAP1-knockdown results in cytokinesis failure

We next sought to delineate the mechanism(s) through which ArhGAP11A and RacGAP1 suppression impaired BLBC growth. Unlike in the presence of the apoptosis-inducing compound staurosporine, depletion of ArhGAP11A or RacGAP1 from SUM149 or HCC1937 cells did not result in detectable levels of cleaved PARP1, a marker for cell death (Fig. 2G and H). Hence, increased apoptosis was not responsible for the proliferation defects that arose upon knockdown of ArhGAP11A or RacGAP1.

RacGAP1 has a well-documented role in regulating cytokinesis (29,30), and ArhGAP11A has also recently been implicated in the control of this process (31). We therefore examined the effect of GAP-knockdown on the efficiency of BLBC cell division. Fluorescence microscopy of Hoechst- and phalloidin-stained cells was used to identify bi- or multinucleated cells, which form upon cytokinesis failure. Consistent with the established role of RacGAP1 in regulating cytokinesis, ~30-40% of SUM149 cells, and ~45% of HCC1937 cells became bi/multinucleated upon RacGAP1-knockdown (Fig. 3A, B, and C). Hence, cytokinesis failure is likely to contribute to the inability of RacGAP1-depleted cells to proliferate. In contrast, knockdown of ArhGAP11A only resulted in ~7-9% of cells becoming bi/multinucleated (Fig. 3A, B, and C). Although this slight defect may partially contribute to growth impairment, it is insufficient to account for the substantial growth defects observed upon depletion of ArhGAP11A (Fig. 2C, D, E, and F).

ArhGAP11A-depleted cells undergo p27-mediated cell cycle arrest

To gain further insight into the cause of proliferation failure in ArhGAP11A- and RacGAP1-depleted cells, we next performed flow cytometry analysis of propidium iodide-stained SUM149 cells to identify whether GAP-depletion caused defects in cell cycle progression. Relative to NS cells, ArhGAP11A-deficient cells accumulated in the G1 phase of the cell cycle (Fig. 4A). Hence, ArhGAP11A is required for efficient cell cycle progression in BLBC cells, indicative of an oncogenic role for this GAP.

To elucidate the molecular mechanisms responsible for causing G1 arrest in ArhGAP11A-depleted SUM149 cells, we determined the expression levels of proteins that regulate G1 to S phase cell cycle transition. Notably, phosphorylation and inactivation of the

RB1 tumor suppressor protein, which promotes G1 to S progression by releasing the inhibition of E2F transcription factors, was dramatically reduced upon suppression of ArhGAP11A expression (Fig. 4B). RB1 phosphorylation is dependent on cyclin-dependent kinases (CDKs) in complex with cyclins D1 and E, but can be prevented by CDK inhibitors such as CDKN2A/p16, p21, or p27. After knockdown of ArhGAP11A, strong induction of expression of p27 was detected in SUM149 cells (Fig. 4B). As neither p21 (Fig. 4B) nor p16 (which is deleted in SUM149 cells (32)) were detected under the same conditions, these results indicate that p27 is the CDK inhibitor responsible for the hypophosphorylation of RB1 and the associated G1 arrest that occurs upon depletion of ArhGAP11A. No substantial changes were observed in the expression levels of any of the other cell cycle regulators that we tested (Fig. 4B). We suggest that p27-mediated arrest is likely to be the major mechanism through which ArhGAP11A-depleted cells fail to proliferate.

RacGAP1-knockdown causes senescence

In contrast to ArhGAP11A, knockdown of RacGAP1 from SUM149 cells did not alter the cell cycle profile relative to control cells (Fig. 4A). Despite this, RacGAP1-depleted cells also exhibited low levels of RB1 phosphorylation (Fig. 4B). In these cells, the CDK inhibitor p21 was upregulated (Fig. 4B), indicating that different pathways are activated in response to the depletion of RacGAP1 as compared to ArhGAP11A. As p21-mediated inhibition of RB1 phosphorylation is a pathway known to induce senescence (33), we next tested to see whether senescence may contribute to the growth defect of RacGAP1-deficient cells. Indeed, SUM149 and HCC1937 cells depleted of RacGAP1 were found to have enhanced levels of senescence-associated β-galactosidase expression (Fig. 4C, D, and E). In contrast, ArhGAP11A-knockdown

did not induce senescence (Fig. 4C, D, and E), providing further support for the distinct roles that ArhGAP11A and RacGAP1 play in BLBC cells: depletion of ArhGAP11A leads to decreased growth via p27-mediated cell cycle arrest, whereas RacGAP1-deficient cells fail to proliferate as a result of the combined effects of cytokinesis failure, p21-induction, and the onset of senescence.

ArhGAP11A and RacGAP1 regulate cell spreading and migration

In addition to determining the consequences of depletion of ArhGAP11A and RacGAP1 on BLBC proliferation, we also examined their roles in regulating cell spreading and migration, two processes known to be reliant on Rho GTPase-dependent cytoskeletal dynamics and important for invasive and metastatic cancer growth (34). Suppression of the expression of either ArhGAP11A or RacGAP1 caused mononucleated SUM149 cells to spread on fibronectin with an approximately 30-50% larger area compared to that of NS control cells (Fig. 5A). Enhanced spreading was also observed on uncoated glass coverslips (Supplementary Fig. S3). As bi/multinucleated cells typically exhibit greatly increased spread areas, these cells were excluded from the spreading analysis. These results indicate that both ArhGAP11A and RacGAP1 are involved of the control of cell spreading, suggesting that one function of these GAPs may be to regulate the cytoskeleton via Rho GTPase signaling. However, the possibility that the spread morphologies of these cells may be secondarily linked to the onset of senescence, particularly in the case of RacGAP1-depleted cells, cannot be excluded.

The effect of ArhGAP11A and RacGAP1 on random migration was assessed using time-lapse microscopy of cells plated on fibronectin. Tracking the movement of individual cells over a 24 h period determined that SUM149 cells treated with ArhGAP11A sh3 had a significantly

(~45%) reduced mean velocity compared to that of NS cells (Fig. 5B). This migratory defect was not observed upon treatment with the ArhGAP11A sh5 construct, most likely reflecting the enhanced efficiency of ArhGAP11A knockdown with sh3 compared to sh5 (Supplementary Fig. S1I). Surprisingly, the mean migration velocity of RacGAP1-depleted cells was 32-58% faster than that of NS cells (Fig. 5B). These results indicate that ArhGAP11A promotes, whereas RacGAP1 inhibits, BLBC cell migration *in vitro*.

RhoA activity is increased upon depletion of ArhGAP11A or RacGAP1

As proliferation, cytokinesis, cell cycle progression, spreading, and migration – processes that we have shown to be affected by ArhGAP11A and/or RacGAP1 in BLBC cells – are known to be directly regulated by Rho GTPases, we next set out to determine which specific Rho GTPases are controlled by these two GAPs. In SUM149 cells, pulldown experiments for active GTPases revealed that RhoA, but not Rac1 or Cdc42, was more active upon depletion of ArhGAP11A than in NS-treated cells, by an average of 43-82% (Fig. 6A and B). This is consistent with ArhGAP11A having catalytic GAP activity toward RhoA, as has been demonstrated using *in vitro* assays (31,35,36). In contrast, RacGAP1 has previously been demonstrated to act as a GAP for Rac1 and Cdc42, but not RhoA *in vitro* (37). To our surprise, Rho GTPase pulldown experiments showed that RhoA activity, but not that of Rac1 or Cdc42, was elevated in SUM149 cells upon depletion of RacGAP1 (Fig. 6A and B). These results suggest that RhoA activity in BLBC cells is usually suppressed by ArhGAP11A or RacGAP1. To assess whether increased RhoA activity is responsible for the proliferation defect of ArhGAP11A- or RacGAP1-depleted cells, we performed clonogenic growth assays in the presence of the ROCK protein kinase-selective inhibitor Y-27632. ROCK is a downstream

effector of RhoA and its inhibition caused a partial rescue of the growth defect of ArhGAP11A- or RacGAP1-depleted cells (Fig. 6C), indicating that efficient proliferation is dependent on RhoA signaling being restricted. Furthermore, transfection of SUM149 cells with a constitutively active RhoA mutant (Q63L) resulted in decreased proliferation (Fig. 6D and E), consistent with observations made with another constitutively active RhoA mutant (G14V) in Swiss3T3 fibroblasts (38). For comparison, constitutively active Rac1 and Cdc42 mutants (Q61L) had no effect on proliferation (Supplementary Fig. S4). These results emphasize that persistently elevated RhoA activity is prohibitive to growth.

ArhGAP11A or RacGAP1 expression elicits oncogenic phenotypes in untransformed cells

To assess the effect of ArhGAP11A and RacGAP1 on transformation, we stably overexpressed these proteins in untransformed immortalized human MCF10A breast myoepithelial cells (Fig. 7A). In MTT assays, expression of ArhGAP11A resulted in enhanced MCF10A proliferation, as did expression of a known oncogene, mutationally-activated KRAS4B(G12V) (Fig. 7B). These data support the idea that ArhGAP11A can act as an oncogene and cancer driver. Interestingly, overexpression of HA-RacGAP1 did not cause an increase in proliferation relative to empty vector control (Fig. 7). It is possible that other signaling components must also be altered for RacGAP1 to enhance proliferation.

We next performed MCF10A acinar formation assays to evaluate the effect of RhoGAP overexpression on mammary epithelial morphogenesis. The expression of oncogenes in MCF10A cells can lead to the formation of disrupted acinar morphology; for example, KRAS4B(G12V) expression resulted in the formation of large multi-acinar structures (Fig. 7C). Acini ectopically overexpressing either ArhGAP11A or RacGAP1 exhibited a disrupted, less

spherical architecture relative to control acini as early as 8 days after plating (Fig. 7C and D). This is consistent with an oncogenic effect for both ArhGAP11A and RacGAP1.

Discussion

Our RNA-Seq analysis of human breast tumors identified high expression of several RhoGAP genes in the basal-like subtype, raising the possibility that these negative regulators of Rho GTPases can act as oncoproteins rather than tumor suppressors in BLBC. To address this, we focused on two RhoGAPs that are highly expressed in BLBC, ArhGAP11A and RacGAP1, and established that they are essential for the proliferation of basal-like cell lines. The mechanisms through which these GAPs promote growth differ, in that ArhGAP11A is required for cell cycle progression, whereas cytokinesis is dependent on RacGAP1. Despite the divergent functions of these GAPs, both inhibit RhoA. Hence, our results not only suggest that RhoGAPs have an oncogenic role in BLBC, but also support recent observations that RhoA may act as a tumor suppressor.

While certain RhoGAPs, most notably DLC1 (14,15), have been characterized and well-validated as tumor suppressors, our findings indicate that this classification is not applicable to all members of the RhoGAP family, as had previously been assumed. Compared to RhoGEFs, RhoGAPs are relatively poorly characterized, particularly with regard to their role in cancer. However, examples of other RhoGAPs having pro-tumorigenic functions in breast cancer do exist, such as ArhGAP35 (39), ArhGAP5 (40,41), and ArhGAP31 (42). These studies, coupled to our own, provide accumulating evidence that the role of the RhoGAP family in cancer is not as straightforward as might be expected from the classical interpretation of Rho GTPases as oncogenes. However, our results appear less paradoxical in light of recent studies that have

identified RhoA mutations in cancer that are consistent with reduced activity of this GTPase (16-21). If reduced RhoA activity is indeed advantageous for cancer cell proliferation, then it follows that regulation by GAPs would be a means of achieving this. Given the diverse functions of RhoA, it is possible that RhoGAPs such as ArhGAP11A and RacGAP1 permit RhoA activity to be decreased in a very precise spatiotemporal fashion, such that RhoA can still perform other roles within the cell. This could potentially explain why missense mutations and deletions of RhoA are relatively rare in breast cancer (<1.5%) (28).

Our results indicate that ArhGAP11A is required for G1 to S phase cell cycle progression in BLBC cells. Indeed, depletion of ArhGAP11A caused G1 arrest associated with increased p27 expression. ArhGAP11A has previously been implicated in the control of cytokinesis, with 18% of HeLa cells failing this process upon its knockdown (31). In SUM149 and HCC1937 BLBC cell lines, we did not observe strong defects in cytokinesis upon depletion of ArhGAP11A, however. This discrepancy may reflect heterogeneity between cancer cell lines, and suggests that ArhGAP11A may have additional, non-cytokinesis-related functions in BLBC. In HCT116 colon cancer cells, ArhGAP11A has been shown to regulate invasion, but knockdown of this GAP was not reported to affect cell proliferation (35). Despite this, depletion of ArhGAP11A reduced tumor growth *in vivo* (35), which would appear to agree with our finding that cells deficient of this GAP had substantial defects in proliferation. High expression of ArhGAP11A has also been reported in colorectal, brain, and lung cancers (35), suggesting that its pro-tumorigenic role may not just be limited to breast cancer. Furthermore, a truncated paralog of ArhGAP11A called ArhGAP11B (which is also highly expressed in BLBC, Fig. 1A and B), has recently been shown to promote neocortex expansion (43), raising the intriguing possibility that pro-proliferative functions may be conserved between these related proteins.

Elevated RacGAP1 expression has also been reported in a variety of cancers, including breast, and its overexpression is frequently correlated to poor patient survival and recurrence (44-49). Its biological function in cancer is poorly understood, however. In BLBC, we found that RacGAP1-depleted cells failed to proliferate as a result of cytokinesis failure, p21-induction, and the onset of senescence. The role of RacGAP1 in regulating cytokinesis is well-established (29,30) and, in BLBC, high levels of this protein may be required to facilitate excessive cell division. The GTPase specificity of RacGAP1 is controversial, with one study suggesting that this GAP could be converted from a Rac1- to a RhoA-specific GAP upon phosphorylation by aurora kinase B (50). Others have refuted this finding, however (37). In BLBC cells, similarly to in squamous cell carcinoma cells (46), knockdown of RacGAP1 caused an increase in cellular RhoA activity. It is possible that RhoA activity may be indirectly altered by the depletion of RacGAP1, for example as a result of mislocalized Ect2 – a RhoA-selective RhoGEF whose localization is otherwise regulated by RacGAP1 (30).

Despite depletion of either ArhGAP11A or RacGAP1 leading to increased RhoA activity, we observed distinct mechanisms of growth suppression in each case. These differences may be caused by GAP catalytic-independent functions, discrete spatiotemporal regulation of RhoA signaling, or through additional GAP activity on other Rho GTPase substrates. The only known catalytic domain found in ArhGAP11A and RacGAP1 is a RhoGAP domain but, through their divergent flanking sequences, they are likely to possess scaffolding functions and interact with distinct sets of proteins. RacGAP1 is a component of the centralspindlin complex during mitosis and cytokinesis, but its function during interphase is not well established. Similarly, little is known about the subcellular localization of ArhGAP11A. In future studies, it would be of interest to characterize the localization of ArhGAP11A and RacGAP1 in BLBC cells to identify

whether this influences their distinct abilities to act as oncogenes. Furthermore, a comprehensive biochemical analysis of their GTPase substrates, beyond the three that are conventionally evaluated (RhoA, Rac1, and Cdc42), would provide additional insight into their GAP-dependent mechanisms of action.

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Figure Legends

Figure 1

ArhGAP11A and RacGAP1 are highly expressed in BLBC**.** A-B, the expression of Rho GEF, GAP, GTPase, and GDI genes in basal-like breast tumors is shown relative to their expression in (A) normal or (B) luminal A tumors, as determined from TCGA RNA-Seq data. The 24 genes with the highest relative expression in the basal-like subtype are shown. C, TCGA RNA-Seq data showing row median centered expression levels of *ARHGAP11A* and *RACGAP1* mRNA in

human breast tumors of different subtype. D, blot analyses for ArhGAP11A and RacGAP1 expression in human breast cancer cell lines of different subtypes. Actin expression was determined to verify equivalent loading of total protein.

Figure 2

ArhGAP11A and RacGAP1 are both required for BLBC proliferation. A-B, representative western blots showing knockdown of ArhGAP11A (*left panels*) and RacGAP1 (*right panels*) in (A) SUM149 and (B) HCC1937 cells. C, representative images of crystal violet-stained clonogenic growth assays with SUM149 (*upper*) and HCC1937 (*lower*) cells, with or without knockdown of ArhGAP11A or RacGAP1. D, quantification of mean colony formation for SUM149 (*upper*, n = three independent experiments performed in triplicate ±SEM) and HCC1937 (*lower*, n = three independent experiments performed in triplicate ±SEM) cells, with or without knockdown of ArhGAP11A or RacGAP1, normalized to NS control. Statistical significance was determined by one-sample t test relative to the NS control. E-F, MTT assays showing mean proliferation of (E) SUM149 and (F) HCC1937 cells, with or without knockdown of ArhGAP11A or RacGAP1, each from two independent experiments performed in triplicate ±SEM. Statistical significance, relative to the NS control, was determined by one-way ANOVA with Dunnett's post-hoc test. G-H, blot analyses showing PARP1 cleavage in (G) SUM149 and (H) HCC1937 cells, with or without knockdown of ArhGAP11A or RacGAP1, or after treatment with 1 μM staurosporine. Actin expression was determined to verify equivalent loading of total protein. Data shown are representative of three independent experiments.

Figure 3

RacGAP1-knockdown results in cytokinesis failure**.** A, representative fluorescent images showing actin (green) and Hoechst (blue) staining of SUM149 cells with or without knockdown of ArhGAP11A or RacGAP1. Scale bar = 20 μm. Arrowheads in merged images indicate binucleated cells. B-C, quantification of the percentage of bi/multinucleated (B) SUM149 or (C) HCC1937 cells with or without knockdown of ArhGAP11A or RacGAP1 (n = three independent experiments in each case ±SEM, ≥ 108 total cells/condition/experiment). Statistical significance, relative to the NS control, was determined by one-way ANOVA with Dunnett's post-hoc test.

Figure 4

ArhGAP11A-depleted cells undergo p27-mediated cell cycle arrest whereas knockdown of RacGAP1 causes senescence. A, shown in the upper panels are representative histograms indicating the fluorescence intensity of propidium iodide (PI)-stained NS- or ArhGAP11A sh3-treated SUM149 cells, as identified using flow cytometry. The relative boundaries of the G1, S, and G2/M phases are indicated. Shown in the lower panel is quantification of the mean percentage of SUM149 cells in each phase of the cell cycle, with or without knockdown of ArhGAP11A or RacGAP1, from three independent experiments performed in triplicate ±SEM. Statistical significance for the G1 category is shown relative to the NS control, and was determined by one-way ANOVA with Dunnett's post-hoc test. B, blot analyses for the indicated proteins, from one experiment, representative of three. Actin expression was determined to verify equivalent loading of total protein. C, representative images of senescence-associated β-galactosidase (β-gal)-stained SUM149 cells (in blue), with or without knockdown of ArhGAP11A or RacGAP1. D-E, quantification of the mean percentage of β-gal-stained (D) SUM149 or (E) HCC1937 cells, with or without knockdown of ArhGAP11A or RacGAP1 (n =

three independent experiments performed in triplicate in each case ±SEM, > 400 total cells/condition/experiment). Statistical significance, relative to the NS control, was determined by one-way ANOVA with Dunnett's post-hoc test.

Figure 5

ArhGAP11A and RacGAP1 regulate cell spreading and migration. A, shown in the upper panel are representative fluorescent images of actin (green) and Hoechst (blue) staining of SUM149 cells, with or without knockdown of ArhGAP11A or RacGAP1, after 2 h on 10 μg/ml fibronectin-coated coverslips. Scale bar = 20 μm. Shown in the lower panel is quantification of mean cell area (n = three independent experiments ±SEM, ≥85 cells/condition/experiment) after spreading on fibronectin, normalized to NS control. Statistical significance was determined by one-sample t test relative to the NS control. Multinucleated cells were excluded from the analysis. B, mean random migration velocity of SUM149 cells, with or without knockdown of ArhGAP11A or RacGAP1, on fibronectin-coated coverslips, as measured from 24 h time-lapse movies. Data shown were pooled from three independent experiments, ≥110 total cell tracks/condition ±SEM. Statistical significance, relative to the NS control, was determined by one-way ANOVA with Dunnett's post-hoc test.

Figure 6

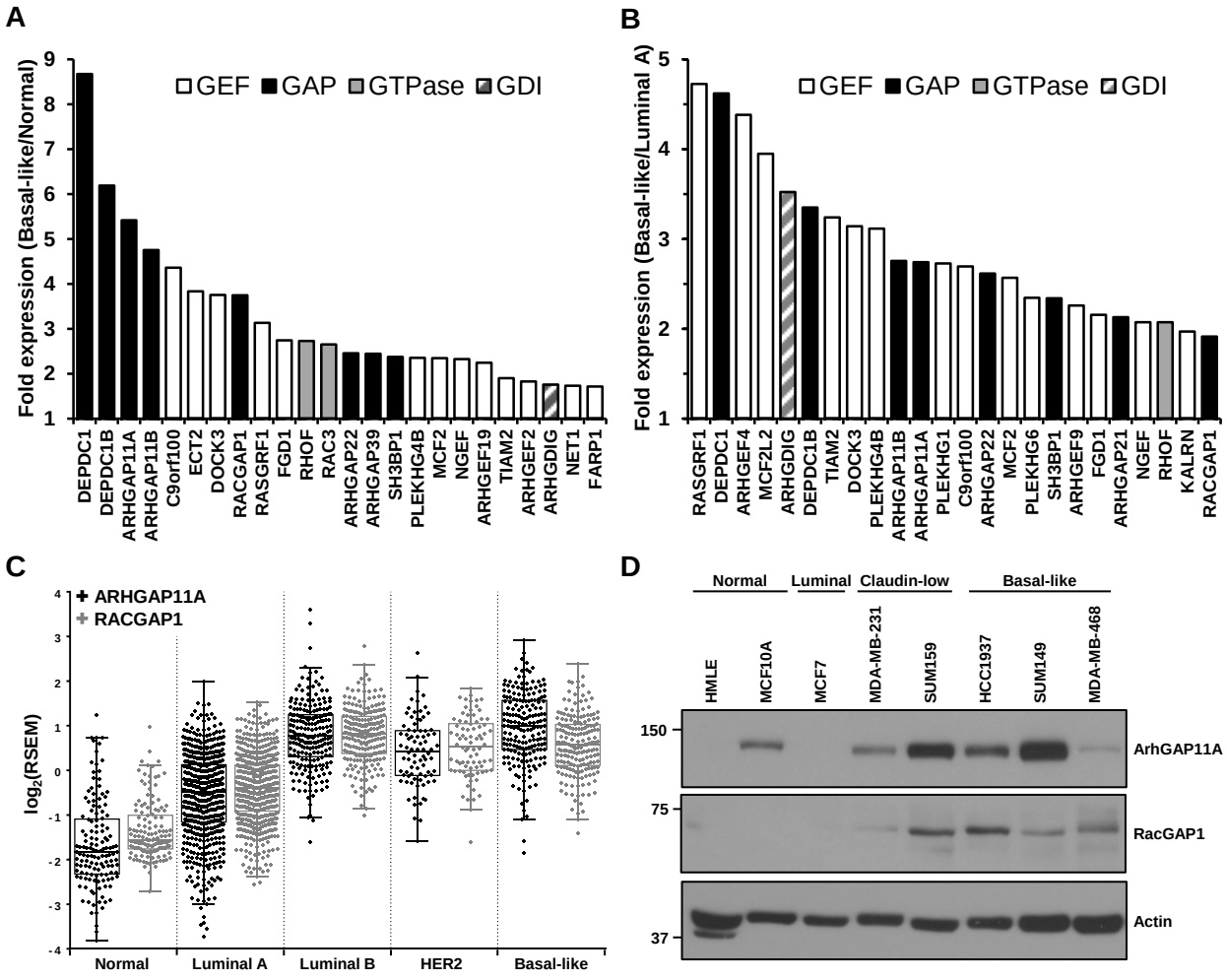
RhoA activity is increased upon depletion of ArhGAP11A or RacGAP1. A, blot analyses for GTP-bound RhoA, Rac1, and Cdc42 levels in SUM149 cells, with or without knockdown of ArhGAP11A or RacGAP1, following Rho GTPase pulldown experiments. Total protein levels were detected from whole cell lysate (*non-specific band from previous blot). Actin expression

was determined to verify equivalent loading of total protein. B, densitometric quantification of the mean ratio of GTP-bound to total RhoA, Rac1, or Cdc42 (±SEM), from seven, eight, or four independent pulldown experiments, respectively. Data were normalized to the NS control in each case. Statistical significance was determined by one-sample t test relative to the NS control. C, quantification of mean SUM149 colony formation, with or without knockdown of ArhGAP11A or RacGAP1, and after treatment with or without 10 μM Y-27632 (n = four independent experiments performed in triplicate ±SEM, normalized to untreated SUM149 cells). Statistical significance between untreated and Y-27632-treated samples was determined by two-tailed t test. D, blot analyses for RhoA showing endogenous RhoA and overexpression of Myc-RhoA Q63L in SUM149 cells. Actin expression was determined to verify equivalent loading of total protein. E, MTT assay showing SUM149 cell proliferation in the presence or absence of Myc-RhoA Q63L (n = two independent experiments performed in triplicate ±SEM).

Figure 7

ArhGAP11A or RacGAP1 expression elicits oncogenic phenotypes in untransformed cells. A, blot analyses for HA-epitope tag (*left panels*), ArhGAP11A (*upper right panels*), and RacGAP1 (*lower right panels*) showing overexpression of HA-ArhGAP11A, HA-RacGAP1, and HA-KRAS4B(G12V) in MCF10A cells. Actin expression was determined to verify equivalent loading of total protein. B, MTT assay to monitor MCF10A proliferation in empty vector control cells or cells expressing HA-ArhGAP11A, HA-RacGAP1, or HA-KRAS4B(G12V) (n = three independent experiments performed in triplicate ±SEM). C, representative images of actin (green) and Hoechst (blue) stained MCF10A acini expressing empty vector control, HA-ArhGAP11A, HA-RacGAP1, or HA-KRAS4B(G12V), after 12 days growth on Matrigel. Scale

bar = 50 μm. D, quantification of relative acini perimeter/area over time for MCF10A cells expressing empty vector, HA-ArhGAP11A, or RacGAP1. Data shown were pooled from two independent experiments performed in duplicate, ≥161 total acini/day/condition ±SEM. Statistical significance, relative to the empty vector control, was determined by one-way ANOVA with Dunnett's post-hoc test.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Fold expression (Basal-like/Normal)
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DEPDC1
DEPDC1B
ARHGAP11A
ARHGAP11B
C9orf100
ECT2
DOCK3
RACGAP1
RASGRF1
FGD1
RHOF
RAC3
ARHGAP22
ARHGAP39
SH3BP1
PLEKHG4B
MCF2
NGEF
ARHGEF19
TIAM2
ARHGEF2
ARHGDIG
NET1
FARP1
B
Fold expression (Basal-like/Luminal A)
GEF GAP GTPase GDI
RASGRF1
DEPDC1
ARHGEF4
MCF2L2
ARHGDIG
DEPDC1B
TIAM2
DOCK3
PLEKHG4B
ARHGAP11B
ARHGAP11A
PLEKHG1
C9orf100
ARHGAP22
MCF2
PLEKHG6
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ARHGEF9
FGD1
ARHGAP21
NGEF
RHOF
KALRN
RACGAP1
C
ARHGAP11A
RACGAP1
log2(RSEM)
Normal
Luminal A
Luminal B
HER2
Basal-like
D
Normal
Luminal
Claudin-low
Basal-like
HMLE
MCF10A
MCF7
MDA-MB-231
SUM159
HCC1937
SUM149
MDA-MB-468
150
75
37
ArhGAP11A
RacGAP1
Actin

Figure 1

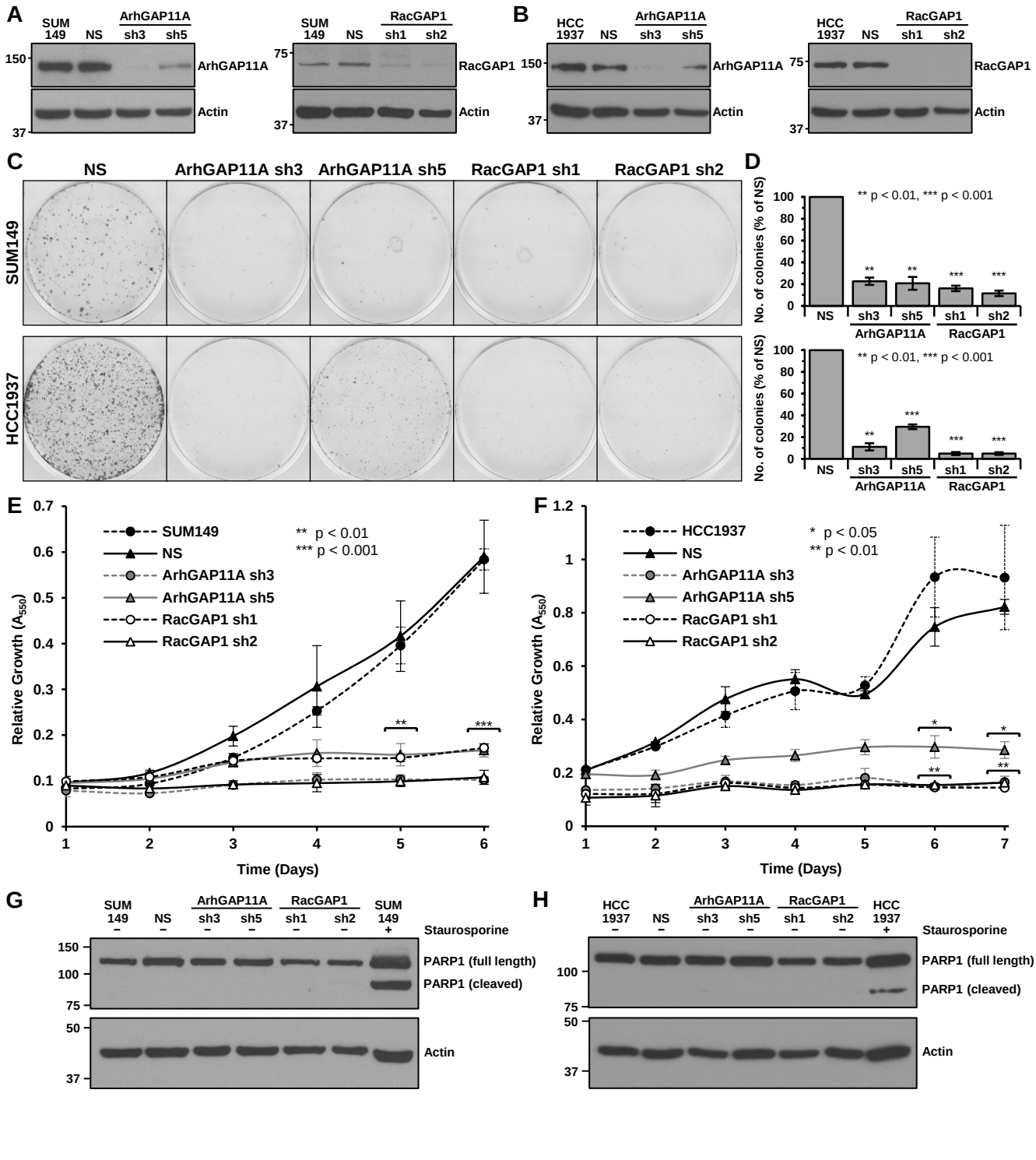
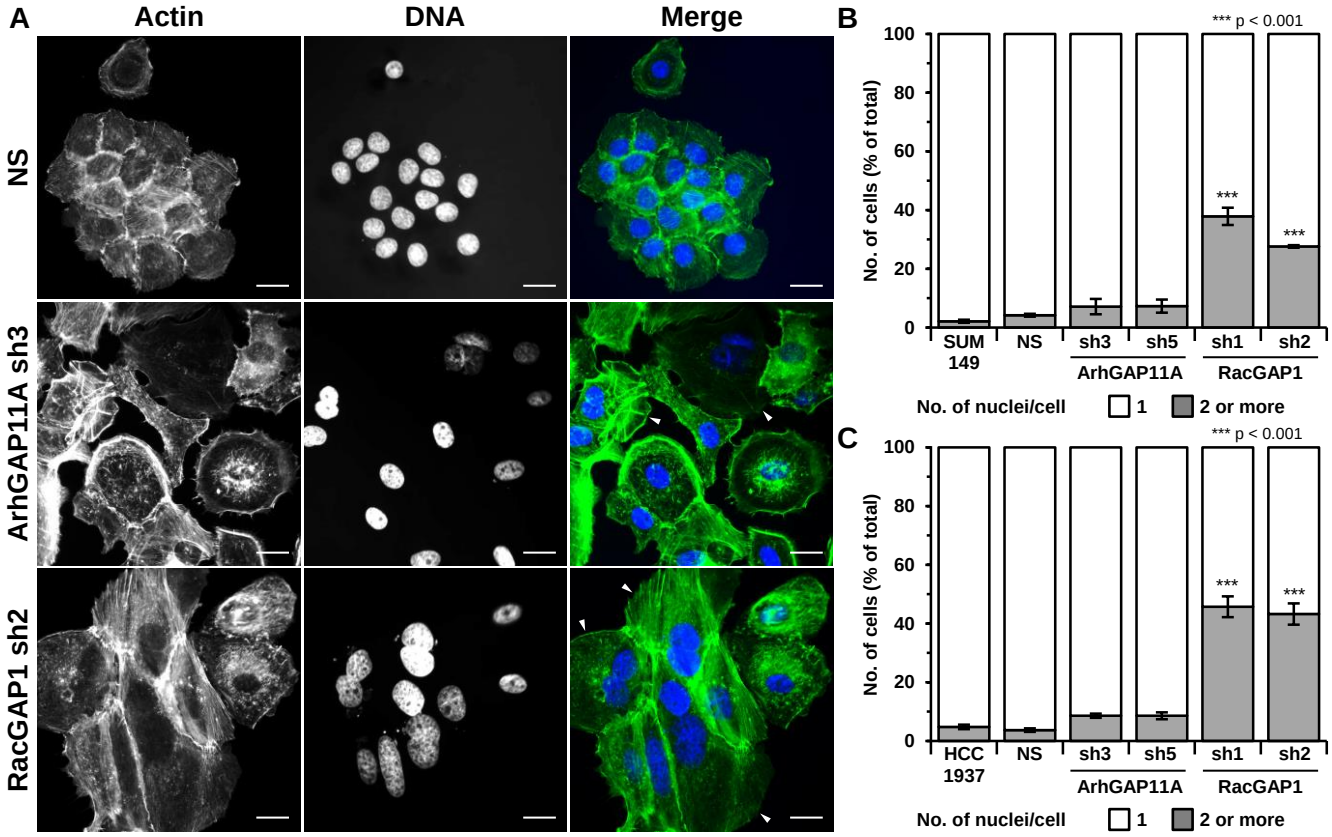

A
SUM 149
NS
ArhGAP11A
sh3
sh5
150
ArhGAP11A
Actin
37
SUM 149
NS
RacGAP1
sh1
sh2
75
RacGAP1
Actin
37
B
HCC 1937
NS
ArhGAP11A
sh3
sh5
150
ArhGAP11A
Actin
37
HCC 1937
NS
RacGAP1
sh1
sh2
75
RacGAP1
Actin
37
C
NS
ArhGAP11A sh3
ArhGAP11A sh5
RacGAP1 sh1
RacGAP1 sh2
SUM149
HCC1937
D
** p < 0.01, *** p < 0.001
No. of colonies (% of NS)
100
80
60
40
20
0
**
**
***
***
NS
sh3
sh5
sh1
sh2
ArhGAP11A
RacGAP1
** p < 0.01, *** p < 0.001
No. of colonies (% of NS)
**
***
***
***
E
SUM149
NS
ArhGAP11A sh3
ArhGAP11A sh5
RacGAP1 sh1
RacGAP1 sh2
** p < 0.01
*** p < 0.001
Relative Growth (A550)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
Time (Days)
F
HCC1937
NS
ArhGAP11A sh3
ArhGAP11A sh5
RacGAP1 sh1
RacGAP1 sh2
* p < 0.05
** p < 0.01
Relative Growth (A550)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
Time (Days)
G
SUM 149
NS
ArhGAP11A
sh3
sh5
RacGAP1
sh1
sh2
SUM 149
Staurosporine
150
100
75
PARP1 (full length)
PARP1 (cleaved)
50
Actin
37
H
HCC 1937
NS
ArhGAP11A
sh3
sh5
RacGAP1
sh1
sh2
HCC 1937
Staurosporine
100
75
PARP1 (full length)
PARP1 (cleaved)
50
Actin
37

Figure 2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Actin
DNA
Merge
NS
ArhGAP11A sh3
RacGAP1 sh2
B
*** p < 0.001
No. of cells (% of total)
100
80
60
40
20
0
***
***
SUM 149
NS
sh3
sh5
sh1
sh2
ArhGAP11A
RacGAP1
No. of nuclei/cell
1
2 or more
C
*** p < 0.001
No. of cells (% of total)
100
80
60
40
20
0
***
***
HCC 1937
NS
sh3
sh5
sh1
sh2
ArhGAP11A
RacGAP1
No. of nuclei/cell
1
2 or more

Figure 3

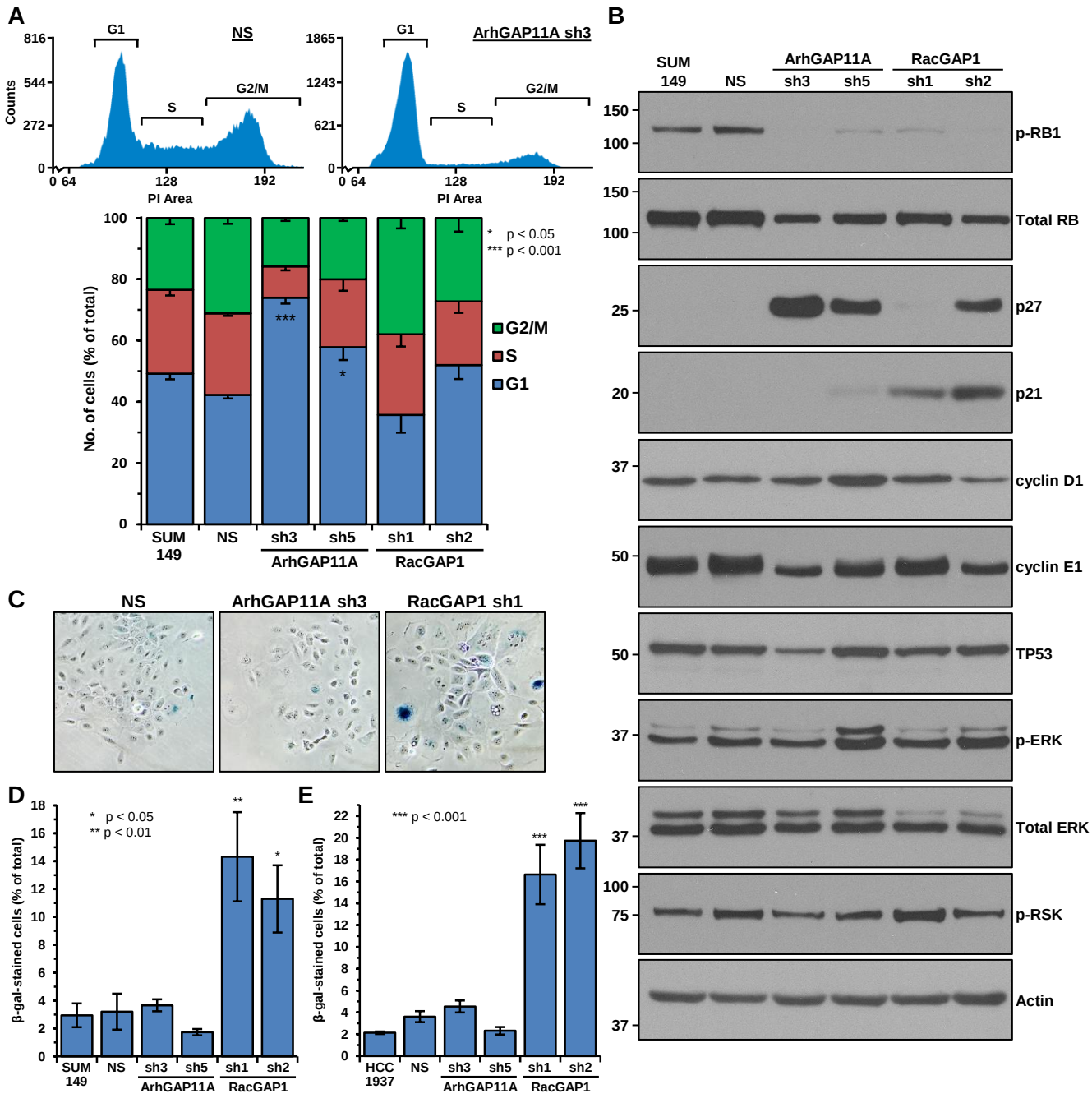

A
NS
ArhGAP11A sh3
G1
S
G2/M
Counts
PI Area
No. of cells (% of total)
* p < 0.05
*** p < 0.001
G2/M
S
G1
SUM 149
NS
sh3
sh5
sh1
sh2
ArhGAP11A
RacGAP1
B
SUM 149
NS
ArhGAP11A
RacGAP1
p-RB1
Total RB
p27
p21
cyclin D1
cyclin E1
TP53
p-ERK
Total ERK
p-RSK
Actin
C
NS
ArhGAP11A sh3
RacGAP1 sh1
D
* p < 0.05
** p < 0.01
β-gal-stained cells (% of total)
E
*** p < 0.001
HCC 1937

Figure 4

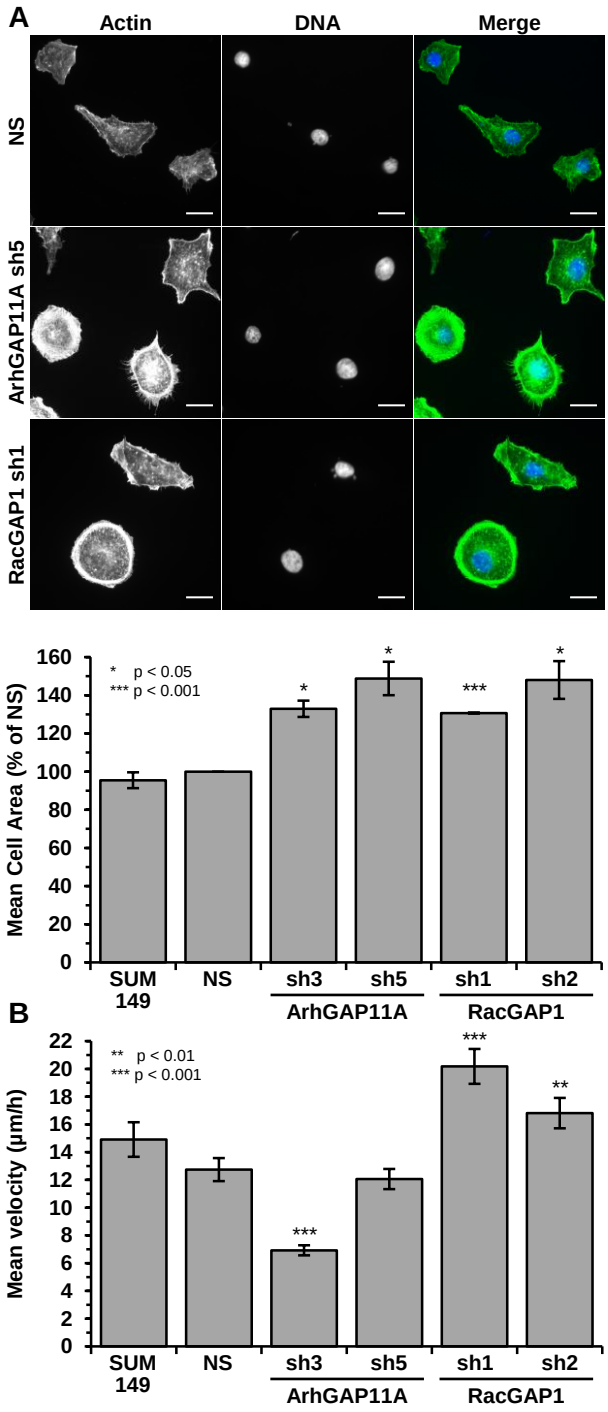

A
Actin
DNA
Merge
NS
ArhGAP11A sh5
RacGAP1 sh1
Mean Cell Area (% of NS)
* p < 0.05
*** p < 0.001
SUM 149
NS
sh3
sh5
sh1
sh2
ArhGAP11A
RacGAP1
B
Mean velocity (µm/h)
** p < 0.01
*** p < 0.001

Figure 5

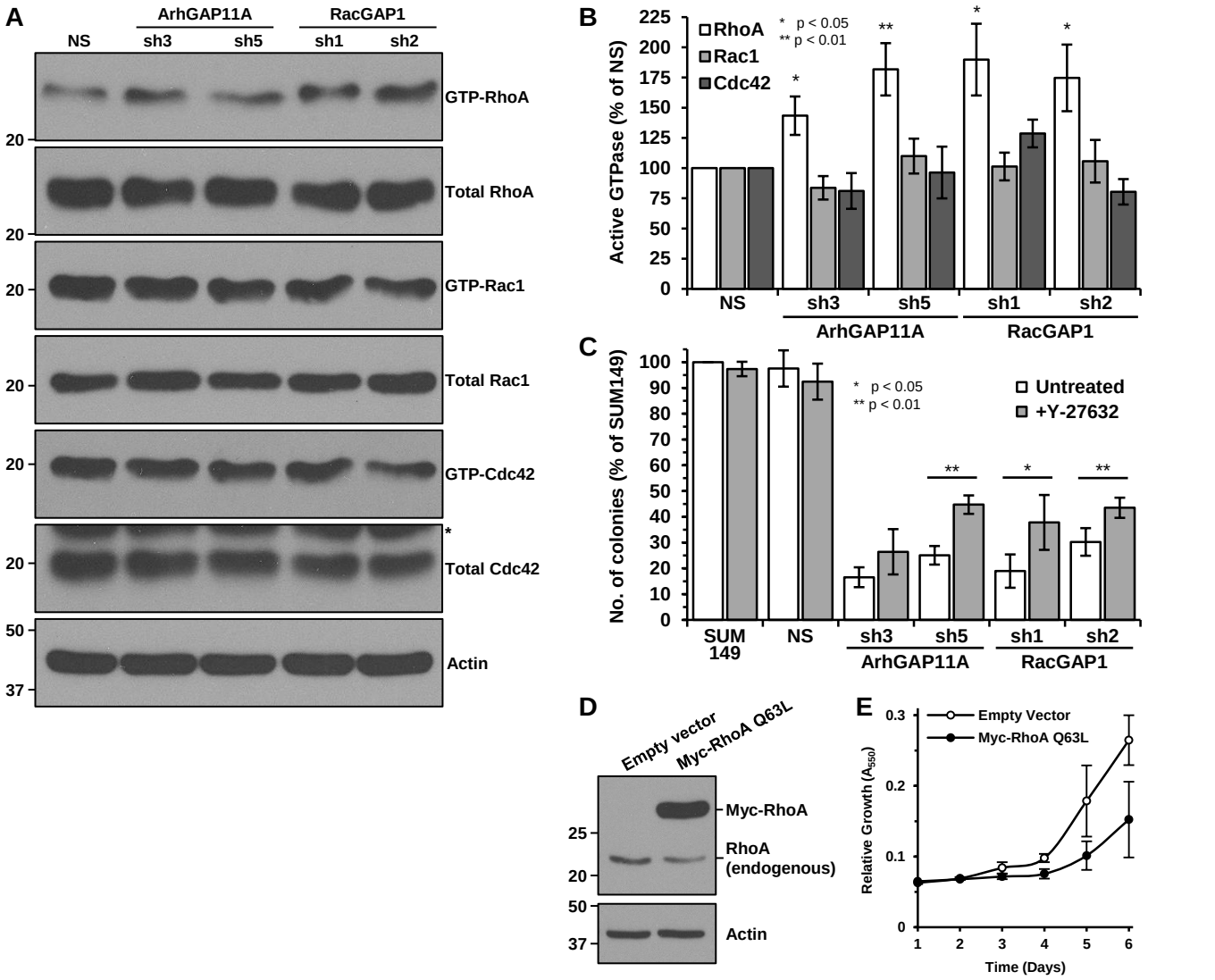

A
ArhGAP11A
RacGAP1
NS
sh3
sh5
sh1
sh2
GTP-RhoA
Total RhoA
GTP-Rac1
Total Rac1
GTP-Cdc42
Total Cdc42
Actin
20
50
37
B
RhoA
Rac1
Cdc42
* p < 0.05
** p < 0.01
Active GTPase (% of NS)
C
No. of colonies (% of SUM149)
Untreated
+Y-27632
SUM 149
D
Empty vector
Myc-RhoA Q63L
Myc-RhoA
RhoA (endogenous)
E
Empty Vector
Myc-RhoA Q63L
Relative Growth (A550)
Time (Days)

Figure 6

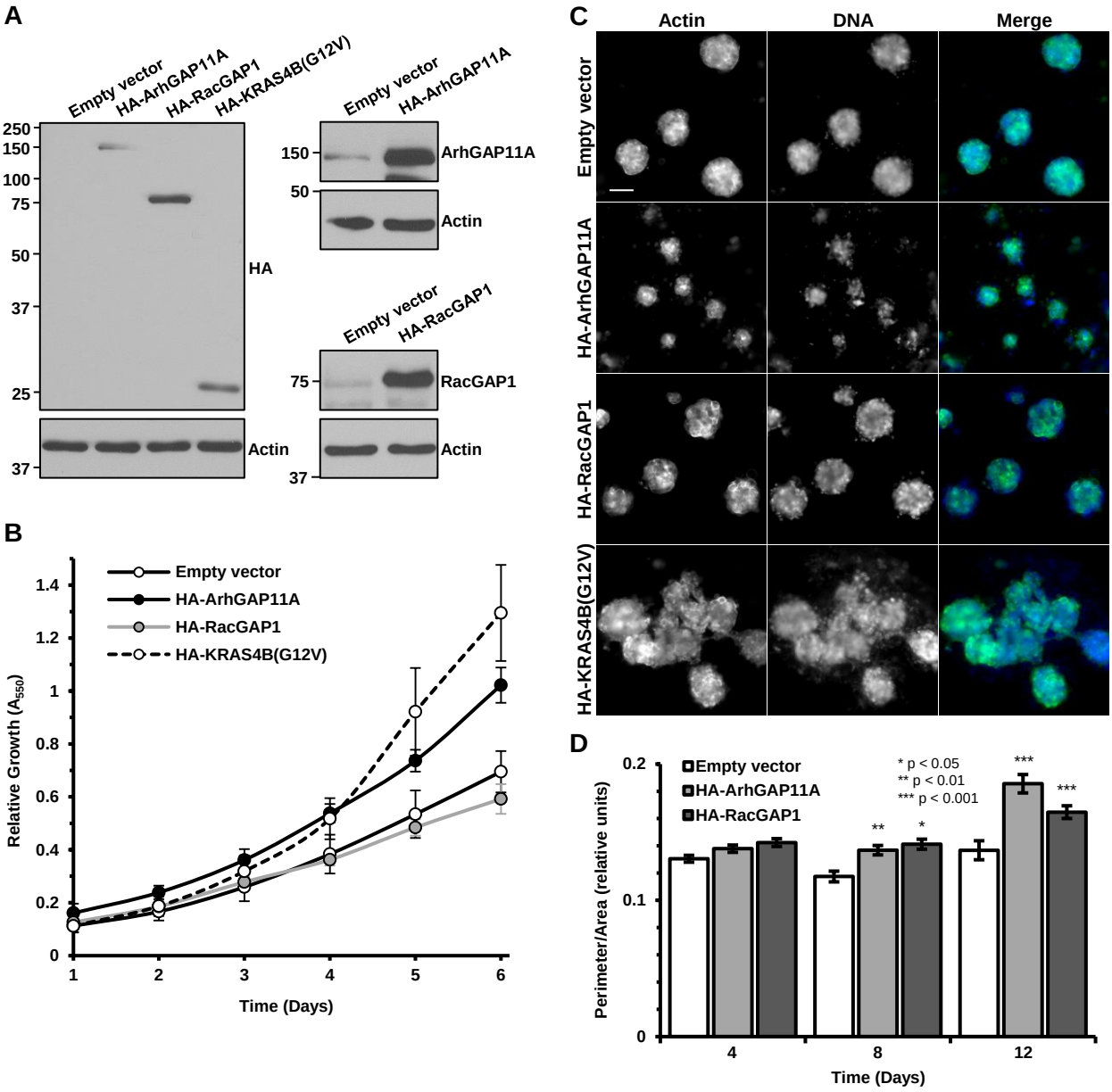

A
Empty vector
HA-ArhGAP11A
HA-RacGAP1
HA-KRAS4B(G12V)
250
150
100
75
50
37
25
HA
Actin
37
Empty vector
HA-ArhGAP11A
150
50
ArhGAP11A
Actin
Empty vector
HA-RacGAP1
75
RacGAP1
Actin
37
B
Empty vector
HA-ArhGAP11A
HA-RacGAP1
HA-KRAS4B(G12V)
Relative Growth (A550)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
Time (Days)
C
Actin
DNA
Merge
Empty vector
HA-ArhGAP11A
HA-RacGAP1
HA-KRAS4B(G12V)
D
Empty vector
HA-ArhGAP11A
HA-RacGAP1
* p < 0.05
** p < 0.01
*** p < 0.001
Perimeter/Area (relative units)
0.2
0.1
0
4
8
12
Time (Days)

Figure 7

Supplementary Figure Legends

Supplementary Figure S1

Western blots showing knockdown of ArhGAP11A or RacGAP1. The panels in this figure correspond to the data presented in the following figures: A, Fig. 2C (SUM149), 3A and B, Supplementary Fig. S3. B, Fig. 2C (HCC1937) and F. C, Fig. 2E, 5A. D, Fig. 2G. E, Fig. 2H, 3C. F, Fig. 4A. G, Fig. 4B, C, and D. H, Fig. 4E. I, Fig. 5B. J, Fig. 6A and B. K, Fig. 6C.

Supplementary Figure S2

ArhGAP11A and RacGAP1 are both required for proliferation of HER2-enriched and luminal B breast cancer cell lines. A, blot analyses for ArhGAP11A (*left panels*) and RacGAP1 (*right panels*) expression in human breast cancer cell lines of the HER2-enriched, luminal B, or BLBC subtypes. B-D, representative western blots showing knockdown of ArhGAP11A (*left panels*) and RacGAP1 (*right panels*) in (B) BT474, (C) MCF7, and (D) T47D cells. Actin expression was determined to verify equivalent loading of total protein. E, MTT assays showing mean proliferation of BT474 (*left panel*), MCF7 (*center panel*), and T47D (*right panel*) cells, with or without knockdown of ArhGAP11A or RacGAP1, after the indicated number of days. Data are from two (T47D) or three (BT474 and MCF7) independent experiments performed in triplicate ±SEM. Statistical significance, relative to the NS control, was determined by one-way ANOVA with Dunnett's post-hoc test.

Supplementary Figure S3

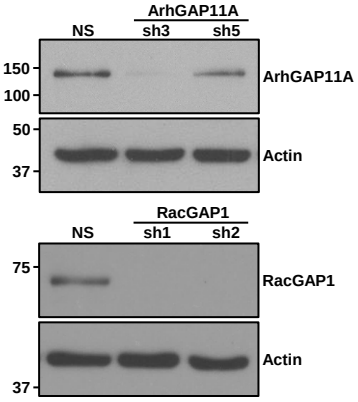
ArhGAP11A and RacGAP1 regulate cell spreading. Quantification of the mean cell area of SUM149 cells (with or without knockdown of ArhGAP11A or RacGAP1) after 72 h on uncoated

coverslips. Data shown are pooled from two independent experiments ±SEM, ≥117 total cells/condition. Statistical significance, relative to the NS control, was determined by one-way ANOVA with Dunnett's post-hoc test. Multinucleated cells were excluded from the analysis.

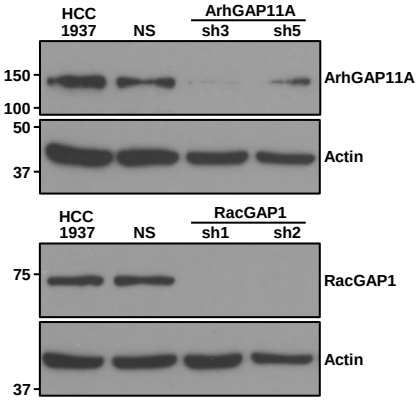
Supplementary Figure S4

Constitutively active Rac1 and Cdc42 do not affect BLBC proliferation. A, blot analyses for Rac1 (*left panels*) and Cdc42 (*right panels*) showing endogenous Rac1 or Cdc42 and overexpression of Myc-Rac1 Q61L or Myc-Cdc42 Q61L in SUM149 cells. Actin expression was determined to verify equivalent loading of total protein. B, MTT assay showing SUM149 cell proliferation in the presence or absence of Myc-Rac1 Q61L or Myc-Cdc42 Q61L (n = two independent experiments performed in triplicate ±SEM).

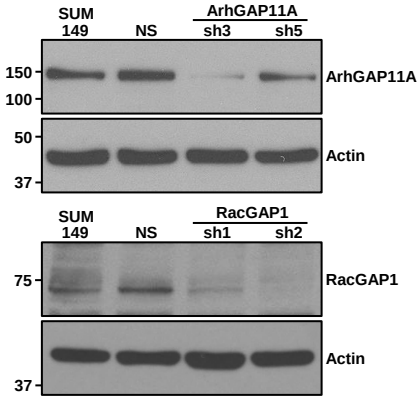
A, Figures 2C (SUM149), 3A-B, and S3



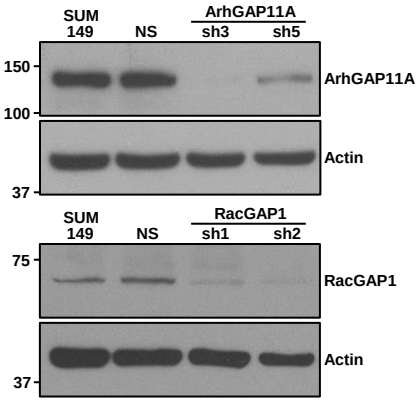
B, Figure 2C (HCC1937), F



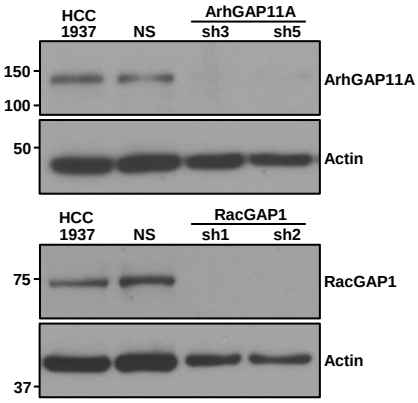
C, Figures 2E and 5A



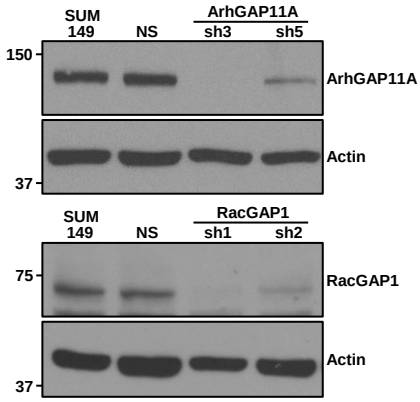
D, Figure 2G



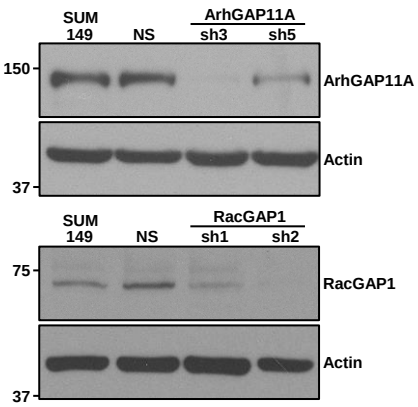
E, Figures 2H and 3C



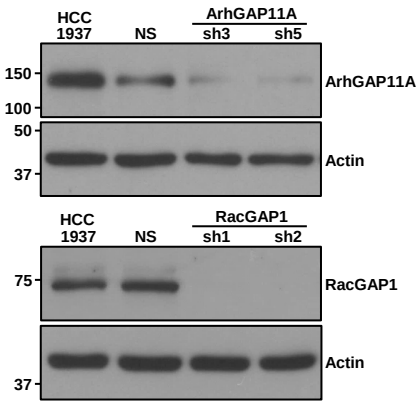
F, Figure 4A



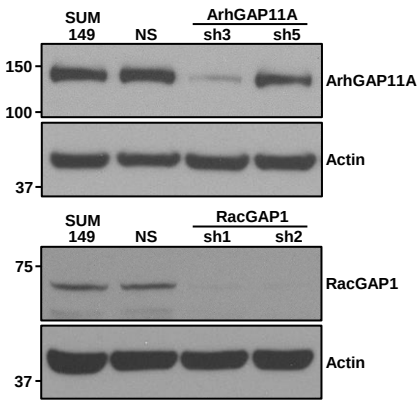
G, Figure 4B-D



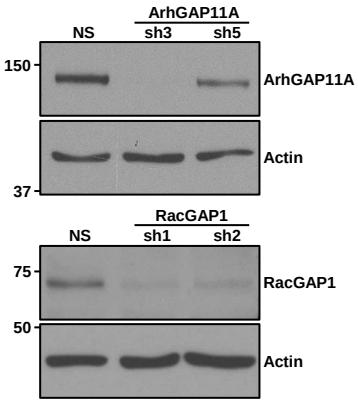
H, Figures 4E



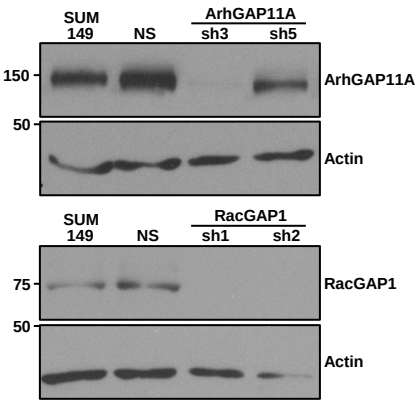
I, Figure 5B



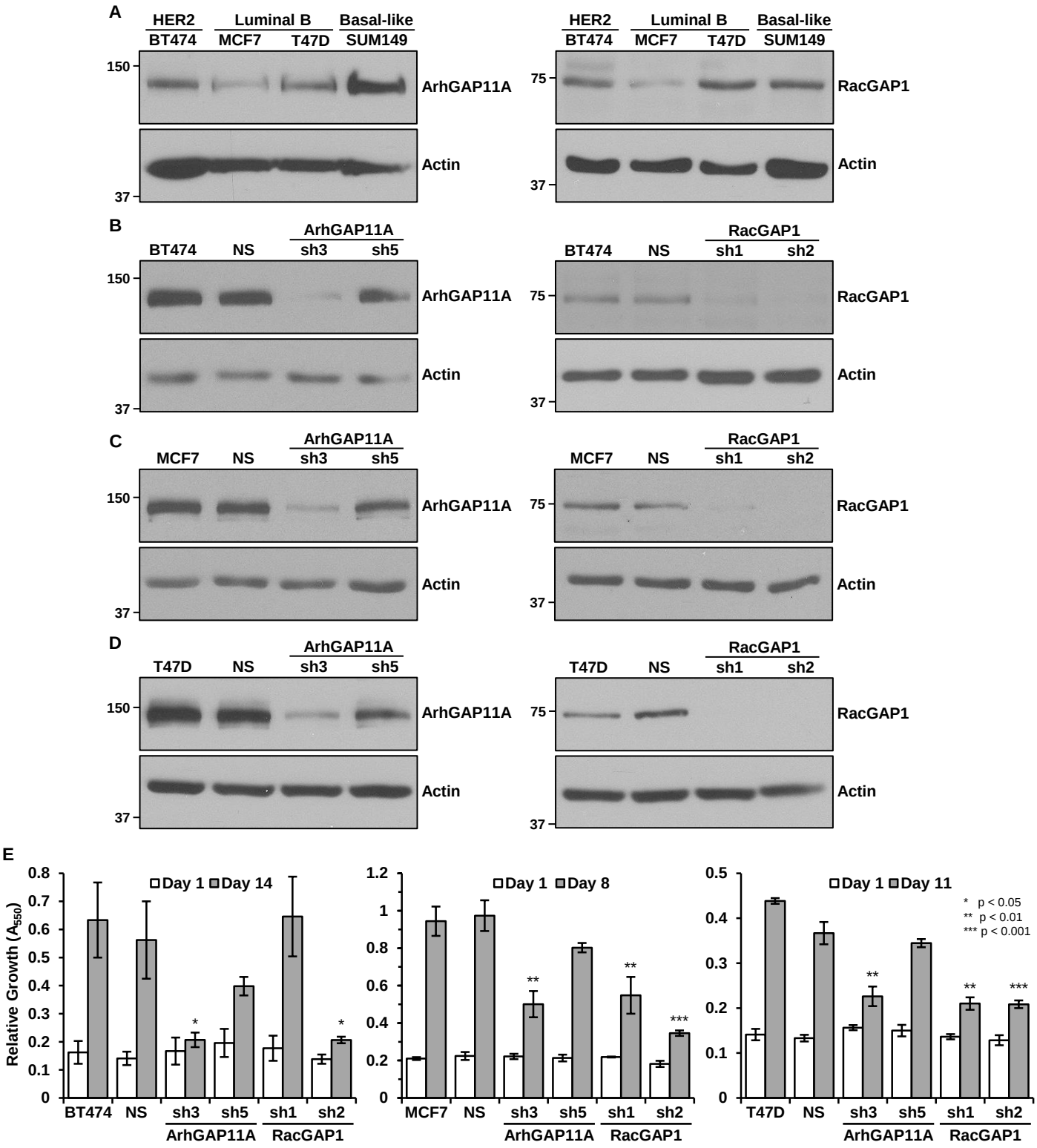
J, Figure 6A-B



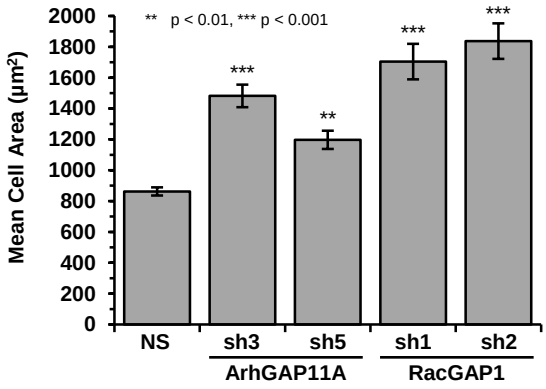
K, Figure 6C



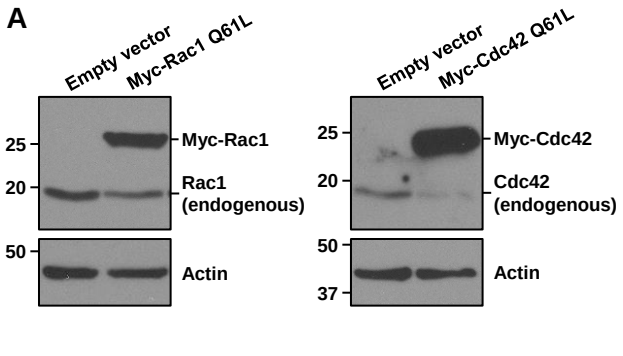
Supplementary Figure S1

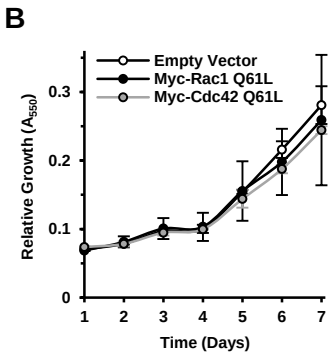


Supplementary Figure S2



Supplementary Figure S3





Supplementary Figure S4

Appendix 2: Acceptance letter from Cancer Research

From: "jennifer.jones@aacr.org" <jennifer.jones@aacr.org>
Reply-To: "jennifer.jones@aacr.org" <jennifer.jones@aacr.org>
Date: Friday, April 22, 2016 at 11:28 AM
To: Channing J Der <channing_der@med.unc.edu>
Cc: Channing J Der <channing_der@med.unc.edu>

Subject: CANCER RESEARCH CAN-15-2923R -- Decision Rendered

Dr. Channing J Der
University of North Carolina at Chapel Hill
Dept. of Pharmacology
Lineberger Comprehensive Cancer Center
CB7295
Chapel Hill, NC 27599-7295

Re: CAN-15-2923R
Rho GTPase Transcriptome Analysis Reveals Oncogenic Roles for Rho GTPase-activating Proteins in Basal-like Breast Cancers

Dear Dr. Der:

I am pleased to inform you that your above-referenced manuscript has been accepted for publication in CANCER RESEARCH.

You will receive your proofs electronically within 2 to 3 weeks. If you expect your e-mail address to change within that time, please notify the CANCER RESEARCH Editorial Office (cancerres@aacr.org). The e-mail you will receive will include detailed information about marking and returning your proofs. Please read, correct, and return proofs within 2 business days. NOTE THAT IF PROOFS ARE NOT RETURNED WITHIN THIS TIMEFRAME, PUBLICATION OF THE ARTICLE WILL BE DELAYED.

Please note that the assignment to which section of the journal your article will appear is made at the Editors' discretion.

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Sincerely,
Rakesh Kumar, PhD
Deputy/Senior Editor
Cancer Research

Reviewer #1 (Reviewer Comments to the Author):

The authors have completed additional experiments and text revisions that have satisfied my concerns.

Reviewer #2 (Reviewer Comments to the Author):

The authors addressed the concerns related to the initial submission.